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THE RELATIVE PROTEIN EFFICIENCY AND THE RELATIVE VITAMIN G CONTENT OF COMMON PROTEIN SUPPLEMENTS USED IN POULTRY RATIONS[1]

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INTRODUCTION

Considerable evidence has accumulated which indicates that a wide variation exists in the growth-promoting properties of the protein-rich feedstuffs used in poultry rations. These discrepancies have been ascribed largely to differences in quality of proteins. Norris and Heuser (*12*)[3] suggested, however, that these properties may be due not only to protein quality but also to vitamin G content. Later Norris and his coworkers (*13*) and Bethke, Record, and Kennard (*1*) showed that the need of poultry for vitamin G is very great and that cereals and their byproducts do not contain sufficient of this vitamin to satisfy the need. It was evident, then, that much of the vitamin G required by poultry was supplied by means of the protein concentrates. Hence the usual conception of the value of these feedstuffs was only partially correct and a further investigation of this field was necessary.

Preliminary studies with chicks to determine the growth-promoting properties of meat scraps, whale-meat meals, and fishmeals, appeared to confirm the suggestion of Norris and Heuser (*12*). Accordingly, the subsequent work was conducted in two phases, based upon the theory that the value of protein-rich feedstuffs for feeding poultry was due both to protein quality and to vitamin G content. The first of these studies was limited to the evaluation of the proteins by determining their relative efficiency of utilization for growth processes. The second dealt with the quantitative determination of the vitamin G content of these materials. In a preliminary report of this investigation (*25*), evidence was presented which supports the initial theory. This has been corroborated recently by Record, Bethke, and Wilder (*18*), who found that haddock meal frequently contains considerable vitamin G as well as protein of fine quality.

THE RELATIVE EFFICIENCY OF THE PROTEINS

METHOD

Originally it was planned to adapt to chicks the method of Mitchell (*10*) for determining the biological value of proteins. St. John and his coworkers (*20*) have since reported on such an adaptation.

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[2] Acknowledgment is hereby made of the cooperation of the Grange League Federation Exchange, Inc., of Ithaca, N. Y., which made this investigation possible by establishing a temporary investigatorship at Cornell University. Acknowledgment is also made of the courtesy of a large number of firms in supplying the writers with many of the samples studied. Space does not permit that they be listed by name.

[3] Reference is made by number (italic) to Literature Cited, p. 398.

However, the lack of a suitable synthetic ration capable of supporting the chicks satisfactorily for even a period of 1 week and the lack of an accurate method for differentiating urinary and fecal nitrogen added such variants to this procedure that the necessary assumptions, in addition to those in the original method, might seriously have impaired the value of the data. Furthermore, the practical application of determinations of biological value is difficult, since the effects of digestibility have been eliminated. The biological value, therefore, expresses only one phase of protein utilization, that indicating the extent to which the assimilated protein under study meets the amino acid requirement of the animal used. A simple nitrogen balance trial was therefore decided upon since the results obtained would be a measure of the entire natural process rather than any part of it and could be directly applied in practice. A practical cereal basal ration was used in these studies.

TABLE 1.—*Constitution of the casein control ration*

Ingredients	Parts	Nitrogen	Fat	Ash
	Percent	*Percent*	*Percent*	*Percent*
Yellow corn meal	33.82	0.511	1.57	0.52
Wheat-flour middlings	20.00	.589	.91	.68
Salt	1.00			1.00
Cod-liver oil	.50		.50	
Liver-extract (dry basis)	.56	.045		.06
Subtotal	55.88	1.145	2.98	2.26
Casein	7.69	1.055	.01	.17
Lard	1.51		1.51	
Bone ash	3.57			3.57
Subtotal	68.65	2.200	4.50	6.00
Sugar	31.35			
Total	100.00	2.200	4.50	6.00

The casein control ration and the method of standardizing the nitrogen, fat, and ash at the required levels are given in table 1. The amount of wheat-flour middlings was standardized at 20 percent but the corn meal varied in different lots of the basal diet according to the amount necessary to make the total nitrogen from these sources 1.10 percent. The only variation between the rations within a trial was in the protein supplement used and in the amounts of bone ash, lard, and sugar necessary to make the proper adjustments. The total amount of nitrogen supplied was equivalent to 13.75 percent of protein ($N \times 6.25$), which is below the optimum requirement of White Leghorn chicks during the early stages of growth (*4*).

The liver extract was obtained by a method suggested by R. M. Bethke, of the Ohio Agricultural Experiment Station. Pork liver was ground, cooked in double boilers, dried, reground, and extracted with ether. The residue from 1 kg of dried liver was extracted five times with 2 l of 20-percent alcohol by weight. The combined filtrates were heated to boiling, filtered, and concentrated in vacuo so that 1 ml of extract was equivalent to 1 g of the original dried liver. Assays showed that this should be used at a level of 2 ml, or 0.56 g on the dry basis, per 100 g of ration in order to supply sufficient vitamin G.

This ration was mixed together with the exception of the cod-liver oil and was granulated by moistening, drying at low temperature, and

grinding to pass a 10-mesh screen. By granulation, the feed wastage was reduced to negligible proportions.

The trials were conducted with normal White Leghorn chicks during the sixth and seventh weeks of age. Chicks of this age are still in a period of rapid growth, but are not in a period of active molt. The excreta, therefore, consisted of feces, urine, and slough of skin and feathers. During the first 5 weeks, the chicks were reared on a complete ration containing an excess of the vitamin G complex. They were then distributed evenly according to weight and sex in individual cages, equipped with feeders especially designed to reduce feed wastage to a minimum (fig. 1). Six chicks per lot were used in these studies since preliminary work had indicated that this number was sufficient for consistent results. The room used for this work was well ventilated and was heated with steam thermostatically regulated to maintain the temperature at approximately 70° F. A time clock was used to insure a 15-hour day.

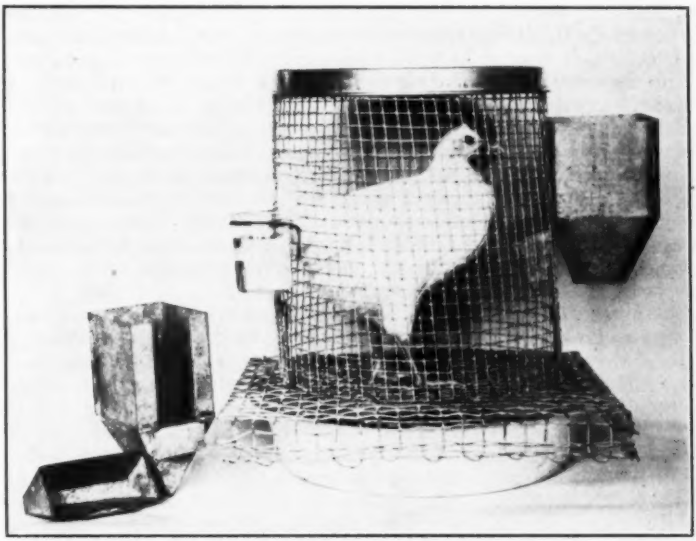

FIGURE 1.—Individual cages, equipped with feeders especially designed to reduce feed wastage to a minimum.

The birds were fed the growth ration for 2 days to accustom them to the cages. They were then given the experimental ration for a preliminary period of 5 days in order to adjust them to the ration and to cleanse the body of residual nitrogen from the growth ration. Preliminary trials had shown that 5 days was adequate for this purpose. During this time the birds were weighed daily at 9 a. m. and were then fed one-half of their daily ration. The remainder was fed at 5 p. m. The amount of feed given each chick daily was equal to 10 percent of the body weight. By this system of feeding the appetites of all chicks, regardless of growth rate, were approximately equally satisfied. This level of feeding was slightly suboptimal.

The experimental period began on the morning of the eighth day and ended at the same time on the fourteenth day. Preliminary studies had shown 6 days to be the optimum length of time. The birds were weighed and were fed as already described except that distilled water was given in place of tap water. Clean cage floors and excreta pans were substituted for those used in the preliminary period as soon as possible after the morning feeding of the eighth day, and the exact hour was noted. In order to prevent loss of ammonia, about 200 ml of distilled water and 10 ml of sulphuric acid were placed in each pan. Several blank pans were likewise treated in order to check the presence of ammonia in the air from outside sources. The birds were removed from the cages on the fourteenth day at the same hour that the excreta pans had been placed in use 6 days previously.

As a general rule, the average gain per bird during this period was about 70 g on the casein control diet. This gradually decreased to about 35 g on diets with a relative protein efficiency of approximately 60. The occasional birds which failed to gain in the same manner as the others in their particular lot or to eat the required amount of feed, or which wasted an undue quantity of feed were eliminated.

The material adhering to the wire-mesh floors was removed into the excreta pans by scrubbing, and the excreta of all birds in each lot left in the experiment were mixed by sex. Concentrated sulphuric acid was poured into each jar of excreta, with constant stirring, to the extent of about 30 ml per bird represented, and the jars were subjected to a temperature of about 60° C. for several days in order that the excreta might undergo partial hydrolysis. The excreta were then rinsed into a tared mixing can, weighed, whipped at high speed in order to thoroughly break up and mix the material, and a sample was weighed out for nitrogen analysis.

The percentage of nitrogen utilized for growth by each sex in each lot was computed from the analyses of the feed consumed and of the excreta, correction being made for any nitrogen found in the blank pans. The percentage storage was then calculated for each sex and the results were averaged by lot. The necessity for doing this was later confirmed by a statistical study by Fisher's method (*3, pp. 105–107*) for paired data of the results of 38 lots on the casein control ration. The average percentage storage for the males was 41.93, and for the females, 40.85. The odds were 49:1 that this difference was not due to chance alone. The relative protein efficiency was determined by dividing the percentage of nitrogen stored from the protein under study by the percentage stored from the casein control lot for that particular trial and multiplying by 100.

TABLE 2.—*Consistency of the results obtained in determining relative protein efficiency of fishmeals*

Fishmeal no.	Trial no.	Protein storage		Relative efficiency		Fishmeal no.	Trial no.	Protein storage		Relative efficiency	
		Sample	Casein	Sample	Average			Sample	Casein	Sample	Average
		Percent	*Percent*					*Percent*	*Percent*		
12	22	45.2	43.2	105	105	31	32	41.8	43.0	97	96
	33	40.7	39.0	104			37	38.2	39.0	98	
13	22	40.4	43.2	94	94		42	41.8	44.4	94	
	33	36.6	39.0	94		32	32	44.3	43.0	103	105
23	33	37.1	39.0	95	95		37	41.9	39.0	107	
	37	37.0	39.0	95			42	46.8	44.4	105	
30	33	42.7	39.0	109	107	51	39	41.9	41.9	100	100
	37 A	38.4	36.6	105			42	44.5	44.4	100	
	42	47.6	44.4	107		52	39	44.2	41.9	105	106
							42	47.2	44.4	106	

Some typical results on fishmeals in which repetitions have been conducted are presented in table 2. These data show that the percentage storage of a particular sample varied somewhat with repetition. An accumulation of such data over a period of several years indicated that this variation was seasonal, tending to be high in the spring and summer and low in midwinter. On the other hand, the results show that this variation was compensated by including the casein pen as a control lot and by expressing the results as relative protein efficiency, since the widest variation in any sample was 4. By using analysis of variance according to Snedecor (*22, pp. 13–20*), the ratio (F) of the mean square between the means of the fishmeals and the mean square within fishmeals was 33.2. Since a value for F of 5.06 under these conditions is highly significant, the chance of getting a difference greater than 4 in the relative protein efficiency by repetition of a determination was less than 1 in 100. This showed that a difference greater than 4 in relative protein efficiency is probably significant, or very nearly so.

Since the standards for fat and bone ash originally set were exceeded slightly from time to time, the effect of variations in these nutrients and, in addition, the effects of adding moderate amounts of fiber and of varying the protein level were studied. The results, shown in table 3, demonstrated that a considerable deviation was permissible in fat, ash, and protein. The addition of fiber in excess of 0.5 percent, however, materially reduced the efficiency of utilization of protein. These data also showed that the level of nitrogen adopted was well under the optimum requirements for birds at this age on restricted feeding despite the fair rate of growth attained.

TABLE 3.—*Effect upon relative protein efficiency of variations in the amounts of various nutrients in the control diet when fed chicks in different pens*

Pen no.	Nutrient in diet				Relative protein efficiency	Pen no.	Nutrient in diet				Relative protein efficiency
	Total nitrogen	Total fat	Total ash	Added fiber			Total nitrogen	Total fat	Total ash	Added fiber	
	Percent	*Percent*	*Percent*	*Percent*			*Percent*	*Percent*	*Percent*	*Percent*	
1	2.20	4.5	6	0	100	6	2.20	4.5	6	.5	101
2	2.20	4.5	8	0	99	7	2.20	4.5	6	1.0	97
3	2.20	6.0	6	0	97	8	2.20	4.5	6	2.0	91
4	2.20	7.0	6	0	97	9	1.92	4.5	6	0	98
5	2.20	7.0	8	0	97	10	2.48	4.5	6	0	97

TABLE 4.—*Relative protein efficiency of common protein supplements*

Protein supplement	Relative protein efficiency of sample—												Average
	a	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*j*	*k*	*l*	
Casein													100
Dried skim milk	100	101											100
White fishmeal:													
Vacuum dried	99	[1] 100	[1] 100	[1] 102	[1] 102	[1] 105	105	[1] 106	[1] 106	[1] 107	107	111	104
Steam dried	[1] 103	[1] 105											104
Flame dried	[1] 94	[1] 95											94
Sardine fishmeal:													
Domestic	94	102											98
Asiatic	89	93											91
Menhaden fishmeal:													
Steam dried	87	88	92	96									91
Flame dried	79	81	[2] 95	[1] [2] 108									80
Soybean meal:													
Expeller process	85	93											89
Hydraulic process	[2] 76	85											85
Meat scrap:													
75 percent protein	67	71											69
60 percent protein	71	80											75
55 percent protein	73	75	78	78	[1] 81	82	83	84	86	86	88	91	82
50 percent protein	67	70	71	72	72	73	75	76	78	[1] 78			73
45 percent protein	72												72
Whale-meat meal:													
70–75 percent protein	73	73											73
55–60 percent protein	54	53											53
Corn-gluten meal	61												61
Ground soybeans	57	60											58

[1] These trials were made with samples produced under experimental conditions.
[2] These trials were not included in the average as the samples were not representative of general commercial production.

EXPERIMENTAL RESULTS

The results obtained on the protein supplements studied are presented in table 4. Dried skim milk was found to possess the same protein efficiency as casein. It appears, therefore, that the cystine deficiency of casein was adequately overcome by the cereal base used.

The proteins of the whitefish meals prepared by vacuum drying surpassed milk proteins. This was surprising in view of the fact that this product is made from the scrap of the cod and haddock fisheries, which consists chiefly of backs and heads. The meals dried by steam under vacuum possessed no advantage over those dried by steam alone, but they were significantly better than the flame-dried ones. This is in accord with the results of Daniel and McCollum (*2*), Maynard and his coworkers (*7*, *8*), and Schneider (*21*). The variation within the vacuum-dried meals was wide. In more detailed studies

of some of these samples, reported elsewhere by the writers (*24*), this variation was found to be due largely to differences in the method of manufacture.

The domestic sardine meals from the Pacific coast were of high protein efficiency. This meal is wet-rendered and then dried in indirect flame or special steam driers which do not burn the meal when properly operated. Since the inclusion of the "stick" (water-soluble materials) had been found to improve the value of whitefish meals (*24*), a sample of concentrated stick obtained in the production of one of the sardine meals was added back to the meal in the correct proportion, with the following results:

Protein efficiency of sardine fishmeal −stick	94
Protein efficiency of sardine fishmeal +stick	89

This drop in value was contrary to results obtained on whitefish meals, but appeared significant since these figures were averages of two trials. The Asiatic sardine meals proved to be slightly inferior to the domestic product.

The results obtained on menhaden fishmeal showed that the samples studied were in general somewhat inferior to the other kinds of fishmeals. Maynard, Bender, and McCay (*7*) and Schneider (*21*) have also noted the inferiority of this product to whitefish meal. Menhaden meal is produced by wet-rendering and is dried generally in direct flame driers, which may cause some charring unless carefully operated. Some producers are now using steam driers. The menhaden meals produced by flame-drying, with two exceptions, were poorer than those produced by steam-drying. One of these better flame-dried meals was a commercial sample which was comparable in appearance and odor to a good steam-dried product. The other was especially prepared for experimental purposes.

A number of these menhaden fishmeals were produced in pairs from comparable raw ingredients by the various methods of drying. The results obtained are as follows:

Protein efficiency of menhaden fishmeal, vacuum-dried	89
Protein efficiency of menhaden fishmeal, flame-dried	81, 108, and 79
Protein efficiency of menhaden fishmeal, steam-dried	96 and 88

The first pair showed a considerable difference in favor of the special vacuum-dried product over the flame-dried one. These data are in accord with those of Maynard and Tunison (*8*) obtained with the rat on the same samples. The second pair of samples was produced in a commercial plant, but the flame drier was started up especially for this purpose, and a small batch was put through at a relatively low temperature and in a shorter time than usual. The results were materially in favor of flame-drying under these conditions. The third pair of samples was from the same plant and was prepared with both driers in commercial production. In this case the results were in favor of steam-drying and substantiate those of Maynard and his coworkers (*7*, *8*) obtained with the rat, and those of Schneider (*21*) obtained with the rat and pig. From these results, it is evident that good menhaden fishmeal can be produced in the direct flame drier only by careful operation.

The soybean meals studied proved to be of slightly lower value than the menhaden fishmeals. The results indicated that meals

produced by the hydraulic process may be as good as expeller meals. The one sample of hydraulic meal that was low had a raw beany flavor, indicating insufficient cooking. The results of Osborne and Mendel (*15*) showed that soybeans must be cooked thoroughly to obtain the best growth in rats. Robison (*19*), using swine, found that not only the soybean but also the soybean meal must be subjected to sufficient heat if satisfactory growth is to be obtained. Further unpublished work by the writers substantiates these results.

Variable but somewhat inferior results were obtained from meat scrap. These samples originated from both rendering plants and large packing houses and were all dry-rendered in steam-jacketed melters. Kraybill (*6*) has described the processes usually followed. This study was restricted mainly to the 50- and 55-percent protein grades, since these represent the bulk of the total production. Despite a wide variation within each grade, there appeared to be a distinct difference in favor of the 55-percent product. The variations in protein efficiency were not found to be correlated necessarily with the bone-ash content of the scraps. Therefore the inferior protein value of 45- and 50-percent protein meat scrap cannot be entirely explained on the basis of higher content of bone proteins. The low efficiency found for the 75 percent protein product is in agreement with the results of Prange, Hauge, and Carrick (*17*). These samples were prepared from pork cracklings, which Hoagland and Snider (*5*) have shown to be a poor source of protein for the growth of rats.

The rendering process apparently causes little variation in the protein efficiency of meat scrap, as is shown by the following samples from table 4 which were prepared under controlled conditions:

	50 percent protein grade	*50 percent protein grade*
Protein efficiency of meat scrap, regular rendered	78	86
Protein efficiency of meat scrap, vacuum rendered	78	81

The two grades were produced by different packers under commercial conditions. Each pair came from comparable raw ingredients, one being rendered in the regular manner and the other with some vacuum. No beneficial effect was noted from the use of vacuum, since in the first pair the temperature used with the vacuum was so low as to greatly increase the time of processing, and in the second pair the temperature and the length of application were about the same as in the regular method. Theoretically there would be no benefits from the use of vacuum unless either the temperature or the time of processing or both were reduced under the usual procedure. This has been substantiated to some extent by results obtained on several pairs of samples from a third company, as follows:

	50 percent protein grade	*55 percent protein grade*
Protein efficiency of meat scrap, regular rendered	67	70
Protein efficiency of meat scrap, vacuum rendered	72	71

These meat scraps were prepared from comparable raw material, apparently of lower inherent value than those just discussed. The vacuum process was a commercial one in which a high vacuum was used, and the time and temperature of processing were both less than in the regular method. The results were slightly in favor of vacuum-

rendering. It is probable, therefore, that the variations in the protein quality of meat scraps are caused largely by differences in the raw materials used, as concluded by Pope (*16*).

Whale-meat meal on the whole proved to be of low protein efficiency, although the two high protein samples ranked with 50-percent meat scrap. No definite information was available as to the method of production of these samples. The two higher grade meals were of South American and South African origin, the other two samples were from the South Georgia Islands.

The low protein efficiency obtained on the samples of soybeans is in agreement with the results of other workers, who found that raw soybeans were an unsatisfactory source of protein for the growth of rats (Osborne and Mendel (*15*)), pigs (Robison (*19*)), and chicks (Tomhave and Mumford (*23*)).

Corn-gluten meal was of low protein efficiency in the basal ration used, but in further work conducted at this station, it has been found possible to supplement corn-gluten meal so as to make it satisfactory.

One sample each of steam-dried blood meal and of vacuum-dried blood flour were included in these studies, but these products rendered the rations so unpalatable that food consumption was barely sufficient for maintenance. Hence, these results are not given. Further work on a practical diet showed that either product could be used to replace meat scrap to the extent of not over 2 percent of the total mash mixture without materially affecting protein efficiency. Hoagland and Snider (*5*), using the rat, and Winter (*26*), using swine, have also found dried blood to be of low protein value and unpalatable.

Sufficient evidence has been accumulated to demonstrate the variations in relative protein efficiency among and within the common protein supplements studied, although the number of samples in some cases was limited. The comparative value of the various classes of protein supplements is in general agreement with the results which Mussehl and Ackerson (*11*) obtained on single samples of some of these materials, using the growth of chicks as a criterion. The variations encountered could be explained not only by inherent differences in the raw materials but also by differences in the processes of manufacture used in producing the finished products. In order to compensate for these unavoidable variations in practice, rations should be formulated with a sufficient excess of protein to provide a margin of safety.

THE RELATIVE VITAMIN G CONTENT

In the first part of this paper the writers have pointed out that wide variations in growth-promoting properties occurred among and within the common protein supplements used in poultry feeding, and data have been presented to show that at least part of these variations was due to differences in relative protein efficiency. Evidence was presented in a preliminary report by Wilgus, Ringrose, and Norris (*25*) that these materials likewise varied in quantity of the vitamin G complex and that this also accounted in part for such differences in growth-promoting properties. In order to verify the preliminary results and to determine more accurately the range in the vitamin G potency of such materials, the relative vitamin G content of most of the samples used in the previous report, as well as of some additional ones, was studied.

METHOD

The assays were made on White Leghorn chicks by a method developed at this laboratory. Chicks were grown on a wire floor to 2 weeks of age on a ration deficient in vitamin G. Preliminary work had shown that this period was adequate to deplete chicks of their natural reserve of this factor. They were then distributed equally in all lots according to weight, the extremes being eliminated. Approximately 70 percent of the chicks originally started were used in the experimental trials. In the seven trials reported here, an average of 20 chicks per lot was used, but the number ranged from 15 to 22 in the different trials.

After distribution, the chicks were individually banded, reweighed, and then fed the basal ration, supplemented by 5 or 10 percent of the materials under study. One lot was continued on the basal ration as a negative control. During the experimental period, records of food consumption and of individual weights were made weekly.

The basal ration was composed of wheat-flour middlings, 20; yellow corn meal, 54; cod-liver oil, 1; purified casein [4] to make 25 percent of protein on the dry basis; bone ash and refined cottonseed oil to standardize the bone ash at 2 percent and the fat at 6 percent in all cases; and cornstarch to make 100 parts. In the supplemented rations, 5 or 10 percent of cornstarch was replaced by the supplement, and the levels of protein, fat, and bone ash were adjusted to the required amounts. In the last two trials, it was necessary to increase the level of bone ash to 3 percent in order to include certain meat scraps high in this respect. No effect of this variation could be noted.

The basal ration used in this investigation was nutritionally complete insofar as known except in the vitamin G complex. It was greatly deficient in the growth-promoting phase of this complex but seldom deficient in the antipellagric phase, and then so slightly that growth was not retarded. On the other hand, the nutritional paralysis associated with this complex (Norris et al. (*14*); Bethke et al. (*1*)) did occur to a moderate extent, but it was not severe enough to affect growth results except in a few cases. Such individuals were eliminated.

In four experiments, the birds were continued on the experimental diets for 6 weeks in order to determine the proper length for the experimental period. Six weeks proved to be too long, as the growth rate slowed up toward the end of that time and the need for vitamin G was reduced. As a result, the birds on the higher levels of vitamin G gained at a proportionately lower rate because of the operation of the law of diminishing increment. The net result was an apparent drop in potency, particularly when the growth on the negative control ration was higher than usual on account of variations in the vitamin G content of the basal ration. This effect, however, was not evident

[4] Commercial casein was purified of vitamin G by dissolving 1 part in 10 parts of dilute sodium hydroxide solution, adjusting the pH to 9.0, and stirring constantly for 16 hours at a temperature of approximately 50° C. The casein was precipitated with hydrochloric acid, drained, washed four times with tap water, and dried.

during the first 4 weeks. The experimental period was limited therefore to 4 weeks.

It was felt that in determining the vitamin G content of protein concentrates the law of diminishing increment might interfere with the interpretation of the results. Hence, a study was made of the effect of this law on feeding graded quantities of vitamin G, dried pork liver being used to supply this vitamin. A straight-line curve

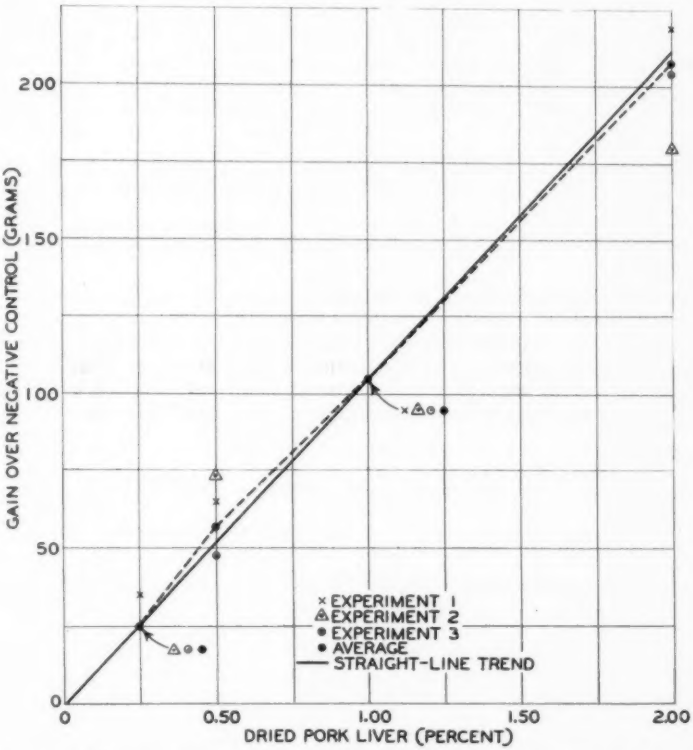

FIGURE 2.—Weight gains of chicks fed dried pork liver as a source of vitamin G over gains of negative control birds.

was found to fit best the data obtained. These data and the straight-line curve are presented in table 5 and figure 2, respectively. This study demonstrated that at the levels of vitamin G used the law of diminishing increment had not become appreciably effective and that, unless unusually large quantities of the protein concentrates were fed, this law would not influence the accuracy of this method of determining the vitamin G content.

TABLE 5.—*Weight gains of chicks fed dried pork liver at different levels as a source of vitamin G over gains of negative control birds*

Dried pork liver fed (percent)	Gain over negative control birds in—							
	Experiment 1		Experiment 2		Experiment 3		Average	
	Gain	Standard error	Gain	Standard error	Gain	Standard error	Gain	Standard error
	Grams		*Grams*		*Grams*		*Grams*	
0.25	34.8	17.69	25.1	11.86	25.0	8.54	26.3	6.45
.50	64.5	12.34	73.2	16.43	47.4	9.93	56.8	6.95
1.00	104.8	19.42	104.8	23.11	105.9	12.81	105.4	9.69
2.00	219.2	8.84	179.8	14.40	203.9	13.64	207.4	6.60

In calculating the data presented in table 5, the mean of the average weight of males and females in each pen and its standard error were obtained by the approximate formula, $\frac{A+B}{2} \pm \frac{\sqrt{a^2+b^2}}{2}$, derived from the exact formula given by Mellor *(9, p. 553)*. This was used in order not to give too much weight to the females, which by nature are lighter than males and therefore have a smaller standard error. Three individuals which lost weight or failed to gain, usually presaging death, were eliminated out of a total of 299 birds. However, in averaging the lot gains made in the three trials on the same level of pork liver, the more exact formula (Mellor *(9)*), $\frac{\frac{A}{a^2}+\frac{B}{b^2}}{\frac{1}{a^2}+\frac{1}{b^2}} \pm \frac{1}{\sqrt{\frac{1}{a^2}+\frac{1}{b^2}}}$, was employed in order to give the greatest weight to the average gain with the smallest probable error, thus giving the greater influence to those lots of chicks in which the least deviation occurred and in which larger numbers were used.

The straight-line curve (fig. 2) was used in determining the relative vitamin G potency of the protein-rich feedstuffs studied. At the end of the 4 weeks' experimental period the average gain per lot over the negative control lot was determined and plotted upon this curve to find the quantity of dried pork liver which gave an equivalent gain. Upon dividing this by the percentage of protein concentrate used and multiplying by 100, the potency in the growth-promoting phase of the vitamin G complex was obtained relative to dried pork liver as 100 percent.

Care was taken to use such levels of the protein concentrates that the gains would fall upon the straight-line curve. In cases in which 10 percent of supplement was added, there appeared to be some possibility that if a supplement of low protein quality were used, the protein efficiency of the diet might be low enough to be a limiting factor. However, in no case was the amount of casein used small

enough to reduce the combined proteins of the corn meal, middlings, and casein to less than 18 percent. Since the results obtained with this type of diet by Heuser and Norris (*4*) indicate that this quantity of protein supports maximum growth, it is improbable that protein efficiency was a limiting factor at any time.

EXPERIMENTAL RESULTS

The relative vitamin G content of the protein supplements used in these experiments is given in table 6. The results in table 5 and those presented in an earlier study (*24*) show that a difference of about 20 g in the gains over the negative control lot was significant when replicate trials were conducted. By interpolating on the straight-line curve shown in figure 2, a gain of 20 g was found to be equal to that produced by 0.20 percent of dried pork liver; therefore a difference in relative vitamin G content of 4, when 5 percent of the material under study was used, or of 2, when 10 percent was used, was significant. It was concluded, therefore, that as a general rule a of difference 5 or more in relative vitamin G content was significant.

TABLE 6.—*Relative vitamin G content of common protein supplements*

Protein supplement	Relative vitamin G content of sample —										Average
	a	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*j*	
Dried pork liver											100
Dried skim milk	17	17	19	20	20						19
White fishmeal:											
Vacuum-dried	[1] 5	[1] 7	[1] 8	8	[1] 9	10	[1] 12	[1] 12	[1] 13	[1] 17	10
Steam-dried	[1] 3	[1] 6									5
Flame-dried	[1] 5										5
Sardine fishmeal:											
Domestic	9	[1 2] 13									9
Asiatic	5										5
Menhaden fishmeal:											
Steam-dried	2	5	6	8							5
Flame-dried	[1] 1	5	6								4
Soybean meal:											
Expeller process	3	4									4
Hydraulic process	3	3									3
Meat scrap:											
75 percent protein	6	5									6
60 percent protein	5	6	10								7
55 percent protein	4	4	5	6	7	7	[1 2] 11	[1 2] 14			5
50 percent protein	3	3	4	5	6	6	7	11	[1] 11	[2] 14	6
45 percent protein	5	7									6
Corn-gluten meal	0										0
Dried blood	0	0									0
Ground soybeans	2	3	3	4							3

[1] These trials were made with samples produced under experimental conditions.
[2] These trials were not included in the average as the samples were not representative of general commercial production.

The value found for dried skim milk was about one-fifth that of dried pork liver. The individual samples possessed potencies within the limits of experimental error. These were all samples representative of commercial production, 4 being roller-dried and 1 spray-dried. The high feeding value attributed to dried skim milk in the past appears to be justified, for the samples studied were uniformly high both in relative protein efficiency and in relative vitamin G content. However, this product is probably of primary value as a source of vitamin G, since some protein supplements nearly equal or even sur-

pass it in protein efficiency when combined with the proteins of corn and wheat (*24*).

The haddock meals possessed about one-half the vitamin G potency of dried skim milk. The vacuum-dried meals were superior to those dried by steam alone or by flame. The variations within the vacuum- and steam-dried samples had previously been shown by the writers (*24*) to be due largely to the method of manufacture and the types of ingredients used. They also showed that the vitamin G content of this kind of fishmeal when properly prepared was sufficient to be of considerable practical importance. This finding is supported by the results of Record, Bethke, and Wilder (*18*).

The domestic sardine meal was comparable with whitefish meal in vitamin G content in spite of the fact that this was a wet-rendered flame-dried product. That this product may have an inherent vitamin G content as high as that of whitefish meal is evidenced by the high value found in an experiment where the stick was returned to the wet-rendered meal in the correct proportion. The results obtained were as follows:

Relative vitamin G content of sardine fishmeal—stick	9
Relative vitamin G content of sardine fishmeal+stick	13

The latter value approximated that of dry-rendered vacuum-dried whitefish meals prepared under proper conditions. This fact indicates that the indirect flame drier used may not have had a more destructive effect on the vitamin G content of the meal than the vacuum type used for whitefish meals. The stick of sardine scrap had the same relative potency as that found for haddock meal in which about one-third of the total vitamin G content of the scrap was found in the stick (Wilgus et al. (*24*)). The Asiatic sardine meal was inferior to the domestic sardine meal in vitamin G content.

The menhaden fishmeals studied were inferior to the other domestic fishmeals. The variations encountered were rather wide and could be only partially explained on the basis of method of manufacture. This was noted in studies of several samples produced in pairs from comparable raw material, the results of which are as follows:

Relative vitamin G content of menhaden fishmeal, steam-dried	5 and 8
Relative vitamin G content of menhaden fishmeal, flame-dried	1 and 6

The difference was in favor of steam-drying, but it was not sufficient to be of practical importance. The values found approximated those of wet-rendered whitefish meals similarly prepared (*24*), which indicates that this fish scrap may be inherently as high in vitamin G as the other varieties studied. Under present methods of production, however, menhaden fishmeal does not appear to be a very dependable source of vitamin G, although it averages about one-fourth the potency of dried skim milk.

The soybean meals were uniformly low in vitamin G. There was no difference between the meals produced by the hydraulic and the solvent process. These possessed the same vitamin potency as the ground soybeans.

The meat scraps, on the whole, were of about the same potency as the menhaden fishmeals. The averages obtained for the various grades were practically identical although there was a considerable range within each grade. The reasons for these variations appear to lie partly in the ingredients used, as all but three were dry-rendered

by the usual process. This is reasonable to suppose because of the nature of the raw materials. On the other hand, some variation may have been due to differences in the temperature of rendering and length of application, since there appears to be no standard procedure in this regard, each producer varying the size of charge, steam pressure, and time to suit his conditions or prejudices. The effect of such variations was noted in a study of paired samples, each pair produced from comparable raw ingredients. These results are as follows:

Relative vitamin G content of meat scrap, regular-rendered	14, 11, 3, 3, and 4
Relative vitamin G content of meat scrap, vacuum-rendered	11, 14, 5, 4, and 7

The first two pairs were made from ingredients containing some livers. In these no beneficial effect was found from the use of vacuum since in the first pair the temperature was so low that the length of application necessary was greatly increased, and in the second pair, the temperature and time were essentially the same as in the regular method. On the other hand, a slight difference in favor of a special vacuum method was noted in the last three samples, although the values were low, due probably to the inherent value of the ingredients. In these three cases, the temperature was considerably lower and the length of application considerably shorter than are usual when the regular method is employed. This fact suggests that the nutritive value of meat scrap may be better preserved by using the smallest amount of heat possible to render it properly. However, the fluctuations in potency found for all the meat scraps studied show that they were not very reliable sources of vitamin G, although they averaged about one-third the potency of dried skim milk.

Neither steam- nor vacuum-dried blood meal nor corn-gluten meal was found to contain any vitamin G.

The results of this study show that most of the products assayed contained variable quantities of vitamin G. This, therefore, confirms the original theory that the growth-promoting properties of this class of feedstuffs may be due to vitamin G content as well as to protein quality. This is further substantiated by the results obtained by Record, Bethke, and Wilder (*18*) on haddock meal. Sufficient samples have been assayed in many cases to indicate the scope of the variations among and within these products. In addition, some samples produced under controlled conditions have demonstrated that these variations may be due not only to the inherent potency of the raw materials but also to the methods of manufacture. The data presented therefore emphasize the importance of using types of raw material of high inherent value, of manufacturing them by methods known to preserve best such value, and of making economic use of the nutritive properties of such products.

SUMMARY

The utilization for growth processes of the protein of protein-rich feedstuffs combined with an equal quantity of protein from yellow corn meal and wheat-flour middlings was determined by means of nitrogen balance trials. Final results were expressed as relative protein efficiency. These values were obtained by dividing the percentage of protein stored from a given ration by normal White Leghorn chicks

during the seventh week of age by that from a standard casein ration, and multiplying by 100.

The relative protein efficiency of a number of the common protein supplements used in poultry rations has been determined. The materials studied, listed in order of their efficiency, are as follows: Vacuum- and steam-dried white fishmeals, 104; dried skim milk, 100; domestic sardine fishmeal, 98; flame-dried white fishmeal, 94; steam-dried menhaden fishmeal and Asiatic sardine meal, 91; soybean meal, 88; flame-dried menhaden fishmeal, 80; meat scrap, 77; whale-meat meal, 64; corn-gluten meal, 61; ground soybeans, 58. These results show that the variations in growth-promoting properties among and within the various classes of common protein supplements studied may be explained at least in part by variations in their relative protein efficiency. These variations were apparently due not only to differences inherent in the raw materials from which these products were obtained, but also to the various factors involved in their manufacture.

A method for determining the relative growth-promoting vitamin G content of feedstuffs has been developed, based upon the gain produced over the control diet by adding 5 or 10 percent of the supplement under study. This was expressed in terms of dried pork liver affording an equivalent gain, and the final results were calculated in percentage with dried pork liver as 100.

A number of samples of common protein supplements used in poultry rations have been assayed by this method. The materials studied, listed in order of their vitamin G potency, are as follows: Dried skim milk, 19; vacuum-dried white fishmeal, 10; domestic sardine fishmeal, 9; meat scrap, 6; steam- and flame-dried white and menhaden fishmeals and Asiatic sardine fishmeal, 5; soybean meal and ground soybeans, 3; corn-gluten meal and dried blood, 0. Thus the growth-promoting properties of the protein concentrates were found in general to be due to vitamin G content as well as to quality of protein. The range within many of the products was found to be rather wide. This was traced to methods of manufacture and types of ingredients used.

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THE NUTRITIVE VALUE OF THE PROTEINS OF CORN-GLUTEN MEAL, LINSEED MEAL, AND SOYBEAN-OIL MEAL [1]

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INTRODUCTION

The results of previous experiments from this laboratory (*22*) [2] indicated no differences in the utilization by growing lambs of the proteins of alfalfa hay and clover hay when fed at a 10-percent level in rations which were presumably adequate in total digestible nutrients, minerals, and vitamins. Furthermore, no differences were observed between the biological value of the proteins of an alfalfa and corn ration and the proteins of a clover and corn ration when both rations were fed at a 10-percent level of protein. The biological values of the proteins of the two latter rations were approximately the same as when each of the hays formed the only source of protein, hence showing no supplementary effect due to the addition of corn protein.

The average of the biological values was 81 for clover protein, 79 for alfalfa protein, 80 for the protein in the combination of clover and corn, and 77 for the protein in the combination of alfalfa and corn. These results were interpreted to indicate that alfalfa hay and clover hay are probably not deficient in quality of protein for sheep when fed in a balanced ration as regards protein and total digestible nutrients.

Since there were no significant differences in protein utilization in these experiments, the question was raised in the minds of the writers as to whether or not there are any actual differences in the nutritive value of the proteins of common feeds for sheep, when the feeds in question are fed in rations which furnish sufficient of total digestible nutrients and other known dietary essentials, and when fed at the same level of protein intake. Very few data have been presented which show any marked differences in the efficiency of protein utilization when common feedstuffs have been fed to ruminants in well-balanced rations (*5*, *6*, *7*, *11*, *19*). The common explanation given is that the ruminant has the ability to synthesize certain of the essential amino acids as a result of bacterial action in the digestive tract. This possibility has been frequently pointed out in the literature, especially with reference to the synthesis of cystine by sheep.

These experimental results (*22*) have suggested the desirability of obtaining further data on the importance of the quality of protein for ruminants and of ascertaining whether differences in the biological value of proteins can be obtained with the sheep using the nitrogen-balance type of experimentation. Therefore, experiments have been conducted to determine the nutritive values for sheep of the proteins

[1] Received for publication May 3, 1935; issued December 1935.
[2] Reference is made by number (italic) to Literature Cited, p. 411.

in three common protein-rich feeds which, on the basis of origin and general nutritive value, should furnish proteins differing in quality.

MATERIALS

Soybean-oil meal was selected as one of the few plant protein supplements that furnish proteins of high quality. Though some experiments have indicated that soybean proteins are rather low in cystine (*17, 21*), they apparently supply more adequate amounts of the essential amino acids than most single seeds or seed byproducts.

In experiments with swine or rats the proteins of soybean-oil meal would probably rank above those of linseed meal and corn-gluten meal. Linseed meal is a popular protein concentrate, but some experiments have shown that its protein is not of the highest quality (*10, 15, 19*). Corn proteins, especially those of the endosperm, are low in two of the essential amino acids—tryptophane and lysine. Corn-gluten meal is probably even lower than the entire corn grain in tryptophane and lysine, as it commonly includes none of the germ the proteins of which are of better quality than those of the endosperm. The immediate object of this investigation, therefore, was to determine what differences, if any, would be found in the nutritive value of the proteins of soybean meal, linseed meal, and corn-gluten meal for growing lambs.

EXPERIMENTAL PROCEDURE

In experiments with swine or rats the proteins of soybean-oil meal tuted for part of the starch and sugar in a low-nitrogen ration and fed to growing wether lambs. The nitrogen balances were determined on each ration, and also the digestibility of the protein, the storage of protein, and the biological value of the proteins (*12, 13*).

The percentage composition of feeds used in this experiment is shown in table 1.

TABLE 1.—*Percentage composition of feeds used*

Feed	Dry matter	Ash	Crude protein	Ether extract	Crude fiber	Nitrogen-free extract
Corn-gluten meal	93.95	2.80	42.36	1.68	4.96	42.15
Linseed meal	93.35	5.11	39.01	5.56	7.44	36.23
Soybean-oil meal	93.05	5.66	42.60	5.73	5.72	33.34

The soybean-oil meal was an expeller-process product and had a pleasant cooked odor and nutlike taste. All the feeds were apparently of good quality and were fed as obtained from the feed manufacturer.

Each of the feeds was added to a low-nitrogen ration of ground wheat straw, cellulose, cornstarch, sugar, corn oil, and salt mixture in such amounts as to provide a protein level of about 11 percent. Of this 11 percent protein, 10 percent came from the feed in question and 1 percent from the straw and other ingredients. The same amount of straw was included in all the rations to furnish bulk, and different amounts of pure cellulose [3] were included to equalize the concentration of crude fiber. Raw cornstarch, cane sugar, and corn oil were included in order to increase and equalize the total energy

[3] Washed sylphrap.

content of the rations. The composition of the rations is given in table 2.

TABLE 2.—*Percentage composition of experimental rations*

Constituent	Low-nitrogen ration	Corn-gluten-meal ration	Linseed-meal ration	Soybean-oil-meal ration
Corn-gluten meal		23.6		
Linseed meal			25.7	
Soybean-oil meal				23.5
Straw, wheat	25.0	25.0	25.0	25.0
Cellulose, regenerated	10.5	9.7	9.0	9.5
Starch	28.0	17.4	17.2	18.0
Sugar	28.0	17.3	17.1	18.0
Corn oil	4.5	4.0	3.0	3.0
Salt mixture	4.0	3.0	3.0	3.0
Total	100	100	100	100
Protein content (N×6.25) [1]	1.15	10.99	11.0	10.96

[1] These percentages of nitrogen represent the averages of analyses on 3 mixes of the low-nitrogen ration and 6 mixes of each of the other 3 rations.

The salt mixture was similar in composition to the one designed by Woodward and McCay (*23*) which is used in this laboratory in synthetic diets for herbivorous animals. The lambs were allowed free access to common salt during all preliminary and intervening periods. A vitamin A and D concentrate was added during experiment 2. It was fed at the rate of 0.0125 g per kilogram of live weight per day. No vitamin B supplements were added because this would have involved adding nitrogen to the ration. The importance of and necessity for vitamins in short balance experiments of this type do not seem to be definitely known.

Three young growing wethers were used. They were purebred Shropshire lambs from the university flock. The details involved in carrying out the experiments and the methods of analysis used were the same as those employed in the previous experiments (*22*). The experimental periods were 10 days in length. The preliminary and intervening periods were also 10 days in length, except those preceding the low-nitrogen periods, which were generally longer.

The lambs were fed twice a day. The plan followed in feeding each lamb on the different rations is shown in table 3:

TABLE 3.—*Plan followed in feeding each lamb the different rations in experiments 1 and 2*

EXPERIMENT 1

Lamb no. 6	Lamb no. 7	Lamb no. 8
Low nitrogen	Low nitrogen	Low nitrogen.
Corn-gluten meal	Linseed meal	Soybean-oil meal.
Linseed meal	Soybean-oil meal	Corn-gluten meal.
Soybean-oil meal	Corn-gluten meal	Linseed meal.
Low nitrogen	Low nitrogen	Low nitrogen.

EXPERIMENT 2

Low nitrogen	Low nitrogen	Low nitrogen.
Soybean-oil meal	Corn-gluten meal	Linseed meal.
Linseed meal	Soybean-oil meal	Corn-gluten meal.
Corn-gluten meal	Linseed meal	Soybean-oil meal.
Low nitrogen	Low nitrogen	Low nitrogen.

The same low-nitrogen period was used for the last period of experiment 1 and for the first period of experiment 2. The second experiment was, therefore, a repetition of experiment 1 with the reversed order of feeding the different rations to each lamb.

DISCUSSION OF RESULTS

The complete results of the metabolism experiments are presented in tables 4 and 5. For convenience of study, the final values are summarized in table 6.

Considerable variations in individual values are noted in a few cases, but they are no greater than commonly occur in experiments of this type. The average results for each of the two trials agreed quite closely in almost all respects. Further repetition of this work and experiments with other species would be desirable.

There was little difference in the average digestibility of the proteins of the corn-gluten-meal and the soybean-oil-meal rations. The proteins of both these rations, however, were slightly more digestible than the proteins of the linseed-meal ration.

There were some differences in the amounts of nitrogen retained or stored on the different rations. The storage from the soybean-oil-meal ration was significantly higher than from either of the other two rations. The average storage of nitrogen was 33.8 percent for the soybean-meal ration, 26.7 percent for the linseed-meal ration, and 26.5 percent for the corn-gluten-meal ration. The odds (*9*) are 302 to 1 against the difference in protein storage between soybean-oil meal and linseed meal being due to chance alone. For the other comparison, the odds were 32 to 1 in favor of soybean-oil meal as compared with corn-gluten meal. There were similar differences in the percentages of digested nitrogen stored. The average percentages of digested nitrogen stored were 51 for soybean-oil meal, 39.8 for corn-gluten meal, and 41.5 for linseed meal. These results clearly show a superiority of the proteins furnished by soybean-oil meal.

The biological values of the protein in the soybean-oil-meal ration were slightly higher than those for either corn-gluten meal or linseed meal. The average was 72.8 percent for soybean-oil-meal proteins, 65.7 for the corn-gluten meal, and 67.7 percent for linseed-meal proteins. The difference between the biological values of soybean meal and of linseed meal is significant as shown by odds of 87 to 1 against this difference being due to chance. A tendency toward statistical significance is also shown in comparing the soybean-oil-meal ration with the corn-gluten-meal ration. The odds in this case are 20 to 1 in favor of the soybean-oil-meal ration.

The proteins of linseed meal were less digestible than corn-gluten-meal proteins but they were utilized a little more efficiently on the average. Soybean proteins were digested at approximately the same rate as the corn-gluten-meal proteins, but were more efficiently utilized as indicated by the storage and biological values of the proteins. These results, as a whole, indicate that soybean-oil meal furnishes a more efficient combination of amino acids than does either linseed meal or corn-gluten meal.

The data on digested protein (nitrogen) stored show greater differences in favor of the soybean-oil-meal proteins than do the biological values. When the metabolic and endogenous nitrogen losses are

considered, and the biological values of the protein computed, the differences in the average values between the soybean-oil-meal ration, and the other two rations becomes less. Since the two methods of evaluating the proteins differ only in the respect that metabolic and endogenous nitrogen are considered in the calculation of biological values, these data may question the accuracy of estimating these nitrogen losses. The writers recognize that the low-nitrogen ration used is not entirely satisfactory for sheep and for most other species. With prolonged feeding of the low-nitrogen ration there is a diminished appetite and falling off in food consumption. The data from the low-nitrogen feeding periods show that as the feed consumption decreases the rate of metabolic nitrogen excreted per unit of feed intake will increase, thus producing an error in the calculation of the biological values. This error, however, may not be very great.

Reduced feed consumption during a period of low-nitrogen feeding will leave an animal in an abnormal condition and may possibly alter the reliability of the results in the following experimental periods. These experiments were conducted in such a manner that each ration was fed to two lambs just following a low-nitrogen period and then again just preceding a low-nitrogen period. Without a single exception, the data show that higher biological values for each feed were obtained immediately following the periods of low-nitrogen feeding. These observations are not in agreement with those of Mitchell (*14*) who believes that a period of low-nitrogen feeding will exert no appreciable effects on the utilization of protein in subsequent experimental periods. There is the possibility, however, that the preliminary period of protein feeding was not of sufficient length before the collection periods were started in these experiments. In no case, however, was there a preliminary period of less than 10 days in length.

The biological values obtained in these experiments for linseed-meal proteins compare favorably with those reported by Mitchell and Hamilton (*15*). Working with swine, these investigators obtained values averaging 61 when the proteins were fed at a 9-percent level. Braman (*2*), working with rats, obtained much higher values, averaging 78 at an 8-percent protein level. Bethke and his associates (*1*) reported average values of 71 for linseed-meal proteins fed to rats at a 10-percent level. There is possibly a slight species difference in in the utilization of proteins which may account for some of the differences obtained, but they can be attributed in part to the difference in level of protein intake.

It should also be pointed out that some dried yeast was provided in the rations used in these investigations (*1, 2, 15*) which undoubtedly furnishes protein of high quality. This protein might supplement that from the feeds in question.

For soybean-oil-meal proteins fed with corn silage and corn to lactating cows, Holdaway, Ellett, and Harris (*8*) reported utilization values of 77 percent. Mitchell and Villegas (*18*) reported average biological values of 64 for the proteins in soybeans fed to rats at a 10-percent level. These values, however, are not directly comparable to those obtained in the experiments reported in this paper. Many practical feeding experiments have been conducted, however, which show the high value of soybean-oil meal as a protein-rich supplement.

TABLE 4.—*Nitrogen metabolism data showing the digestibility and biological value of proteins in the first experiment* [1]

Wether no.	Body weight			Food intake	Dry-matter intake	Nitrogen intake	Fecal nitrogen	Estimated metabolic nitrogen	Food nitrogen in feces	Absorbed nitrogen	Nitrogen in urine	Endogenous nitrogen in urine	Food nitrogen in urine	Food nitrogen utilized	Digestion coefficient	Total nitrogen stored	Digestible nitrogen stored	Biological value
	Initial	Final	Average															
	Kilogram	*Kilogram*	*Kilogram*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Percent*	*Percent*	*Percent*	*Percent*
LOW-NITROGEN RATION																		
6	19.40	20.37	19.89	3,678	3,561.9	6.31	19.78	[2] 0.555	.....	.....	8.30	[2] 0.417	.....	.....	.....	.....	.....	.....
7	19.60	17.30	18.45	3,573	3,458.6	6.45	23.78	[3] .688	.....	.....	8.60	[3] .466	.....	.....	.....	.....	.....	.....
8	16.20	14.87	15.54	2,000	1,938.0	3.39	12.47	[3] .643	.....	.....	9.17	[3] .590	.....	.....	.....	.....	.....	.....
CORN-GLUTEN-MEAL RATION																		
6	22.87	23.90	23.39	6,997	6,655.5	123.01	41.19	36.67	4.52	118.49	40.35	9.01	31.34	87.15	67	34	51	74
7	28.47	29.03	28.75	8,000	7,582.4	142.40	51.47	41.85	9.62	132.78	54.26	9.06	45.20	87.58	64	26	40	66
8	22.43	23.33	22.88	8,000	7,706.4	139.68	41.84	47.63	[4] .00	139.68	64.53	10.75	53.78	85.90	70	24	34	61
LINSEED-MEAL RATION																		
6	23.90	24.37	24.14	7,000	6,664.7	122.64	50.97	36.46	14.51	108.13	47.56	8.62	38.94	69.19	58	20	34	64
7	23.10	23.77	23.44	8,000	7,603.2	141.12	54.54	48.13	6.41	134.71	46.24	9.49	36.75	97.96	61	29	47	73
8	24.27	25.43	24.85	8,000	7,595.0	142.16	46.01	46.03	[4] .00	142.16	53.84	10.19	43.65	98.51	68	30	44	69
SOYBEAN-OIL-MEAL RATION																		
6	26.33	26.80	26.57	7,000	6,606.6	122.22	40.38	35.94	4.44	117.78	40.11	8.74	31.37	86.41	67	34	51	73
7	26.10	27.00	26.55	8,000	7,660.8	140.40	49.06	45.35	3.71	136.69	43.74	9.56	34.18	102.51	65	34	52	75
8	19.87	20.77	20.32	8,000	7,622.4	141.68	40.95	48.10	[4] .00	141.68	45.17	10.77	34.40	107.28	71	39	55	76

LOW-NITROGEN RATION

6	25.83	23.57	24.70	3,511	3,313.2	7.69	17.88	[4] 0.540	------	------	7.39	[3] 0.269	------	------	------	------
7	29.23	27.83	28.53	4,630	4,369.7	10.31	22.26	2.509	------	------	7.66	3.268	------	------	------	------
8	25.30	24.00	24.65	4,000	3,778.8	8.74	22.42	2.593	------	------	8.54	3.346	------	------	------	------

[1] Totals are for 10-day experimental periods.

[2] Fecal nitrogen per 100 g dry matter in feed. These values were used in estimating the metabolic nitrogen in the feces in the experimental periods. The change in the ratio of metabolic nitrogen to dry matter consumed from the first to the last periods was assumed to occur in a linear fashion.

[3] Urinary nitrogen per kilogram of body weight. These values were used in estimating the endogenous nitrogen in the urine in the experimental periods, the same assumption of a linear variation being made as in the case of metabolic nitrogen in the feces.

[4] Estimated metabolic nitrogen greater than fecal nitrogen, therefore it was assumed that no food nitrogen was present in the feces.

TABLE 5.—*Nitrogen metabolism data showing the digestibility and biological value of proteins in the second experiment*[1]

Wether no.	Body weight			Food intake	Dry-matter intake	Nitrogen intake	Fecal nitrogen	Estimated metabolic nitrogen	Food nitrogen in feces	Absorbed nitrogen	Nitrogen in urine	Endogenous nitrogen in urine	Food nitrogen in urine	Food nitrogen utilized	Digestion coefficient	Total nitrogen stored	Digestible nitrogen stored	Biological value
	Initial	Final	Average															
	Kilograms	*Kilograms*	*Kilograms*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Grams*	*Percent*	*Percent*	*Percent*	*Percent*
LOW-NITROGEN RATION																		
6	25.83	23.57	24.70	3,511	3,313.2	7.69	17.88	[2] 0.540	.....	.....	7.39	[3] 0.299	.....	.....	.....	.....	.....	.....
7	29.23	27.83	28.53	4,630	4,369.7	10.31	22.26	[2] .509	.....	.....	7.66	[3] .268	.....	.....	.....	.....	.....	.....
8	25.30	24.00	24.65	4,000	3,778.8	8.74	22.42	[2] .593	.....	.....	8.54	[3] .346	.....	.....	.....	.....	.....	.....
CORN-GLUTEN-MEAL RATION																		
6	33.80	34.90	34.35	8,000	7,426.4	138.88	44.66	37.43	7.23	131.65	61.38	10.72	50.66	80.99	68	24	35	62
7	31.93	33.47	32.70	8,184	7,774.3	144.70	50.44	39.73	10.71	133.99	48.72	7.78	40.94	93.05	65	31	48	69
8	29.00	30.77	29.89	9,000	8,487.0	158.76	57.38	58.22	[4] .00	158.76	69.91	9.59	60.32	98.44	64	20	31	62
LINSEED-MEAL RATION																		
6	31.03	32.10	31.57	8,000	7,432.8	142.64	47.12	38.13	8.99	133.65	48.44	9.76	38.68	94.97	67	33	49	71
7	37.43	37.70	37.57	8,200	7,651.4	140.63	49.02	39.25	9.77	130.86	62.78	7.78	55.00	75.86	65	21	31	58
8	27.87	28.83	28.35	9,000	8,567.1	158.76	62.39	56.03	6.36	152.40	54.13	9.33	44.80	107.60	61	27	44	71
SOYBEAN-OIL-MEAL RATION																		
6	28.33	30.27	29.30	8,000	7,596.0	141.20	51.38	39.65	11.73	129.47	35.59	8.97	26.62	102.85	64	38	60	79
7	34.87	35.93	35.40	8,200	7,662.9	143.91	45.79	39.23	6.56	137.35	57.27	7.89	49.38	87.97	68	28	42	64
8	31.43	32.67	32.05	9,000	8,458.2	155.43	51.92	60.65	[4] .00	155.43	56.28	10.00	46.28	109.15	67	30	46	70

LOW-NITROGEN RATION

6	34.53	31.97	33.25	4,000	3,827.6	6.61	18.92	[2] 0.494			10.52	[2] 0.316						
7	38.70	37.63	38.17	4,750	4,545.3	7.85	23.35	[2] .514			7.31	[2] .192						
8	28.17	29.13	28.65	3,850	3,684.1	6.36	27.82	[2] .755			8.65	[2] .302						

[1] Totals are for 10-day experimental periods.
[2] Fecal nitrogen per 100 g dry matter in feed. These values were used in estimating the metabolic nitrogen in the feces in the experimental periods. The change in the ratio of metabolic nitrogen to dry matter consumed from the first to the last periods was assumed to occur in a linear fashion.
[3] Urinary nitrogen per kilogram of body weight. These values were used in estimating the endogenous nitrogen in the urine in the experimental periods, the same assumption of a linear variation being made as in the case of metabolic nitrogen in the feces.
[4] Estimated metabolic nitrogen greater than fecal nitrogen, therefore it was assumed that no food nitrogen was present in the feces.

TABLE 6.—*The digestibility and biological value of the proteins of corn-gluten meal, linseed meal, and soybean-oil meal when fed to lambs*

Item	Percentage digestibility and biological value when fed to lamb no.—						Average
	6 in experiment—		7 in experiment—		8 in experiment—		
	1	2	1	2	1	2	
Corn-gluten meal:							
Apparent digestibility	67	68	64	65	70	64	66.3±0.67
Percentage nitrogen intake stored	34	24	26	31	24	20	26.5±1.41
Percentage digested nitrogen stored	51	35	40	48	34	31	39.8±2.23
Biological value	74	62	66	60	61	62	65.7±1.40
Linseed meal:							
Apparent digestibility	58	67	61	65	68	61	63.3±1.08
Percentage nitrogen intake stored	20	33	29	21	30	27	26.7±1.42
Percentage digested nitrogen stored	34	49	47	31	44	44	41.5±2.01
Biological value	64	71	73	58	69	71	67.7±1.55
Soybean-oil meal:							
Apparent digestibility	67	64	65	68	71	67	67.0± .67
Percentage nitrogen intake stored	34	38	34	28	39	30	33.8±1.19
Percentage digested nitrogen stored	51	60	52	42	55	46	51.0±1.76
Biological value	73	79	75	64	76	70	72.8±1.45

The values for corn-gluten meal may, in a general way, be compared with those reported for corn grain since the gluten is largely zein, which comprises about 60 percent of the protein of corn grain. Mitchell and Kick (*16*) reported an average biological value of 54 for corn protein when fed to pigs at a protein level of approximately 8 to 9 percent. Later experiments with pigs by Mitchell and Hamilton (*15*) gave corn proteins an average biological value of 61 when fed at a protein level of 8.66 percent.

It should be pointed out that there is a possibility that the small amounts of wheat-straw nitrogen may have supplemented any amino acid deficiencies which any of the three feeds used in this investigation may have. As determined, however, the average values for soybean-oil meal are fairly high. There are no indications of marked amino acid deficiencies in this feed for sheep. These values are of special interest, since sheep have a high requirement for cystine and soybeans have been reported as deficient in this amino acid. However, Csonka and Jones (*3, 4*) have presented analytical data which do not support the idea of a quantitative deficiency of cystine in soybeans. They do admit however, the possibility of a deficiency in quality of protein, due to low availability of amino acids when the raw meal is fed. Osborne and Mendel (*20*) had earlier found the nitrogen of commercial soybean cake and of soybean meal cooked in water was utilized somewhat better than was the case with the raw and dry-heated meals. These writers believed the utilization of protein was increased by making the amino acids more available for assimilation. Perhaps expeller-process soybean meal would not show the cystine deficiencies which raw soybeans have apparently shown in nutrition experiments (*17, 21*).

Some observations on the fleeces of the lambs are of interest. The lambs were shorn at the beginning and again at the end of the experimental work. An examination of the wool fibers of all three fleeces showed "breaks" and distinct weakened places which corresponded exactly with the periods of low-nitrogen feeding. The

breaking strength and diameter of the wool fibers was greatly reduced during the periods of low-nitrogen feeding. The portions of the wool fibers grown during the periods of protein feeding were strong. The wool had a beautiful luster and the fibers were apparently of normal length.

SUMMARY

Metabolism studies were conducted with three growing wether lambs to determine the digestibility, storage, and biological value of the proteins of soybean-oil, corn-gluten, and linseed meals. The experiment was repeated, thus giving six determinations for each feed. Each of the feeds in question was added to a low-nitrogen ration in such amounts as to furnish a protein level of 10 percent, with approximately 1 percent additional being furnished by the other ingredients of the ration. All rations were equalized in energy content.

The average coefficients of apparent digestibility for protein were 67.0 percent for soybean-oil meal, 66.3 for corn-gluten meal, and 63.3 percent for linseed meal.

The lambs were more efficient in storing protein from the soybean-oil-meal ration than from either of the other rations. The average percentage of protein intake stored was 33.8 for soybean-oil-meal, 26.5 for corn-gluten meal, and 26.7 for linseed meal.

Slightly but significantly higher biological values were obtained for the soybean-oil-meal ration. The average of the biological values was 72.8 for soybean-oil-meal proteins, 65.7 for corn-gluten-meal proteins, and 67.7 for linseed-meal proteins.

These data show the superiority of the proteins of soybean-oil meal over those furnished by linseed meal and corn-gluten meal. Furthermore, they indicate that it is possible to measure differences in quality of protein using sheep and the nitrogen-balance type of experimentation.

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NUTRIENT-SOLUTION PURIFICATION FOR REMOVAL OF HEAVY METALS IN DEFICIENCY INVESTIGATIONS WITH ASPERGILLUS NIGER [1]

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INTRODUCTION

The difficulties associated with the demonstration in culture of the chemical elements required by organisms, especially as concerns those necessary in very small quantity, are great enough to make welcome the introduction of any simple procedure whereby an increase in sensitivity or precision may be accomplished. Commercial chemicals, even those of the highest purity, usually contain variable but appreciable traces of impurities, often sufficient in quantity to completely mask the requirement by the organism of the elements present as contaminants. Frequent attempts to improve the purity of commercial chemicals by recrystallization in the laboratory have met with but partial success or with failure. The contradictory and indecisive results often obtained with commercial chemicals and with compounds recrystallized in the laboratory have not aided in establishing definitely the heavy-metal requirements of organisms.

The successful application by the writer (*8*, *9*)[2] of the method of nutrient-solution purification to the study of the heavy-metal requirements of the fungus *Aspergillus niger* Van Tiegh. has rendered desirable a thorough study of the method with a view to obtaining further improvement in results and a better understanding of the principles involved, so as to permit the direct application of the method to similar studies with green plants. The method of purification referred to consists in treating the nutrient solution with calcium carbonate in order to increase its alkalinity and to furnish calcium for the formation of precipitate to adsorb the traces of the heavy-metal precipitates simultaneously formed. In this way the heavy-metal impurities of the nutrient solution are precipitated and removed as a whole. It was found, for example, that the addition of an iron or zinc salt brought about a fivefold increase in yield of the fungus when compounds recrystallized in the laboratory were employed in the nutrient solution, whereas the increase obtained by such additions after the method of nutrient-solution purification with calcium carbonate had been employed was fiftyfold, or approximately 10 times as great.

Analytical methods for the determination of the heavy metals (iron, zinc, copper, and manganese) unfortunately are of insufficient sensitivity or precision to permit of determining the effectiveness of the method of purification. While in exceptional cases, as with iron, these methods permit of the detection of concentrations of about 0.1

[1] Received for publication July 5, 1935; issued December 1935.
[2] Reference is made by number (italic) to Literature Cited p. 424.

mg of cation per liter, the apparent optima of these elements for growth of the fungus are approximately only 0.20, 0.14, 0.06, and 0.03 mg per liter, respectively. Distinct responses in growth that may be duplicated with fair precision are easily obtainable with variations in metal concentration of 0.01 mg of cation per liter. The addition to the nutrient solution of 0.001 mg of zinc per liter (1 part per billion, or 0.05 γ [3] per culture of 50 cc) may bring about an increase of 92.5 percent in yield of *Aspergillus niger*.

The very minute quantities in which these elements are required do not denote their importance to the organism, since the absence of one of the elements from the nutrient solution may result in lowering the yield by 60 to 98 percent. The high dilutions in which these elements seem to be effective perhaps are likewise deceptive, inasmuch as their absorption by the cell may result in their concentration within the protoplast.

Modifications of the writer's method of nutrient-solution purification have been proposed. Bortels' (*1*) studies in effect demonstrate that if the nutrient solution is first made alkaline absorbent charcoal may be substituted for the calcium carbonate in the method of nutrient-solution purification. Bortels obtained results similar to those secured by the writer on the iron and zinc requirements of the fungus, but he included copper also as essential for the organism. Roberg (*7*), on the other hand, was able to show that alkalinity alone is sufficient to precipitate most of the traces of heavy-metal impurities in the nutrient solution. Furthermore, according to Roberg, iron, zinc, and copper are essential to *Aspergillus niger*. Another paper by the writer (*11*), besides confirming the foregoing results with iron, zinc, and copper, presents evidence that manganese also is essential to the mold.

A recent paper by Hopkins (*5*) describes a successful application of the method of nutrient-solution purification for the removal of manganese in studies with algae and with *Lemna*. Growth-manganese ratios of 11 or more were obtained by this author. Instead of using calcium carbonate to increase the alkalinity as well as to supply calcium for the formation of precipitate, Hopkins, like Bortels, employed an alkaline solution but added calcium nitrate instead of charcoal. Emerson (*3*), in his investigations on photosynthesis, found the calcium carbonate procedure helpful in regulating chlorophyll concentration by adjustment of the iron content of the nutrient solution.

Supplementing the present work on the calcium carbonate method of nutrient-solution purification for the removal of heavy metals, data are presented on some of its possible modifications. A limited investigation also was made respecting the possibility of extracting any heavy metals stored in the spores prior to their use for the inoculation of cultures.

METHODS

The methods employed in these studies are similar in all essentials to those previously employed and described. The same "W" strain of *Aspergillus niger* was grown for 6 days, at 34.7° to 35° C., on 50 cc of 5-percent sucrose solution to which the necessary salts had been added, in 200-cc pyrex Erlenmeyer flasks. The incubator employed

[3] $1\gamma = 1\mu g$ (microgram).

has been described elsewhere (*10*). Inoculation was by means of a spore suspension containing 0.5 g of potassium bicarbonate per liter, the inoculum amounting to approximately 0.05 mg of spore material per flask. The cultures were filtered, when harvested, through fritted glass crucibles of porosity 3 and of 35-cc capacity. Instead of drying the felts for 4 days at 103° to 105°, they were dried for 2 hours in a rapid current of dry air at 50° to 70° and then overnight in an oven at 103° to 105°. Compressed air at 60 to 70 pounds' pressure was used instead of a blower and proved satisfactory because of its low relative humidity on expansion. This procedure was found very satisfactory not only in saving time but also in preventing fouling of the crucibles and in preserving the felts in their natural colors.

The nutrient solutions, after treatment and filtration through fritted glass crucibles of porosity 4, were distributed into the Erlenmeyer flasks, and the heavy metals, in solution as sulphate, were added with a pipette to the individual flasks. The flasks then were sterilized by steaming for 10 minutes.

Acidity readings were obtained with a potentiometer and quinhydrone electrode sensitive to 0.01 pH.

NUTRIENT-SOLUTION PURIFICATION WITH CALCIUM CARBONATE

The method of nutrient-solution purification, as employed by the writer (*8*) for the removal of the last traces of iron and zinc from the complete nutrient solution, has several advantages as compared with purification by recrystallization. Instead of a series of operations with each individual compound, the method of nutrient-solution purification requires only two operations and deals with the impurities of the nutrient compounds as a whole. As might be anticipated, it is not only much simpler and more rapid than recrystallization but is also more effective. The decrease in time and in the number of utensils employed is also an important factor in securing absence of contamination and uniformity of results.

The original procedure employed in nutrient-solution purification was to autoclave the complete solution with 15 g of calcium carbonate per liter for 20 minutes at a pressure of 1 atmosphere (120.5° C.). After standing overnight the solution was decanted from the sediment containing the iron and zinc and distributed into culture flasks.

The results of a study of this method for the purpose of determining the effect of the different factors entering into its operation have been tabulated in table 1. The growth ratio, which is determined by dividing the yield obtained in the presence of an element by the yield obtained when this element is omitted, is the reciprocal of the percentage of maximum yield. Duration of treatment has reference to the time elapsing between heat treatment and filtration. The table discloses that variation in the concentration of the nutrient solution or purificant, in the duration of treatment, in the acidity, and in the use of ammonium sulphide did not lead to any marked improvement in results. This is also true in regard to the length of time for which the solution was heated with calcium carbonate and for the separate treatment of sucrose and salts. However, decreasing to a minimum the total amount of nutrient salts per liter led to a slight improvement in results. Filtration immediately following treatment seems advisable. The method, therefore, is not critical with respect to the factors entering into its operation.

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TABLE 1.—*Effect of variations in purification method with calcium carbonate on growth of Aspergillus niger in a nutrient solution containing 5 percent sucrose*

Culture no.	Content in nutrient solution				Duration of treatment	Initial pH	Yield without heavy metals	Growth ratio with—					Maximum individual yield
	NH_4NO_3	KH_2PO_4	$MgSO_4.7H_2O$	$CaCO_3$				Fe	Zn	Cu	Mn	All	
	Grams per liter	*Grams per liter*	*Grams per liter*	*Per-cent*	*Hours*		*Mg*						*Mg*
1	10.0	5.0	2.5	1.5	24	6.64	22.2	12.46	25.09	1.13	1.30	48.93	1,110.9
2	2.1	.75	.55			4.73	208.9	2.15	2.98	1.02	1.66	4.41	936.7
3	2.5	1.0	1.0	3.0	27	7.12	18.6	49.25	16.12	1.76	1.25	55.59	1,056.1
4	2.5	1.0	1.0	1.5	24	6.90	19.4	32.17	17.20	1.48	1.14	46.93	1,000.8
5	2.5	1.0	1.0	1.5	24	6.77	14.2	41.34	34.98	1.67	1.08	67.24	1,040.6
6[1]	2.5	1.0	1.0	1.5	24	7.11	17.5	28.79	27.66	1.39	1.09	48.21	913.8
7[2]	2.5	1.0	1.0	.5	24	6.73	23.1	85.11	23.16	1.54	1.13	40.90	1,007.0
8	2.5	1.0	1.0	.5	24	6.87	10.3	33.50	37.17	1.60	1.20	75.79	822.5
9	2.5	1.0	1.0	.1	24	6.47	17.4	32.03	32.26	1.37	1.24	52.28	1,009.7
10[3]	2.5	1.0	1.0	.1	0	6.29	15.8	62.83	39.21	1.21	2.13	64.03	1,011.6
11[4]	2.5	1.0	1.0	.1	0	5.72	23.2	38.47	36.25	1.14	1.76	45.94	1,078.9
12	2.5	1.0	1.0	.1	48	6.65	16.1	43.35	12.19	1.58	1.49	63.27	1,030.2
13	2.5	1.0	1.0	.1	75	6.63	28.3	30.28	8.98	1.13	1.33	35.52	1,030.7
14[5]	2.5	1.0	1.0	.1	24	6.34	24.1	44.98	17.60	1.17	1.30	43.30	1,043.5
15	5.0	1.0	1.0	.1	26	6.46	19.9	41.90	12.62	1.51	1.37	52.43	1,046.4
16	2.5	2.0	1.0	.1	26	6.32	15.5	43.63	38.15	1.79	1.31	61.08	964.2
17	2.5	1.0	2.0	.1	26	6.49	18.3	80.00	38.82	1.13	1.08	57.27	1,095.3
18[6]	2.5	1.0	1.0	.1	28	6.90	19.4	45.97	35.71	1.33	1.43	49.52	997.5
19	2.5	(7)	1.0	.1	28	6.95	11.8	64.87	50.56	1.10	1.37	87.41	1,039.0
20	2.5	(8)	1.0	.1	28	7.81	12.4	60.57	42.53	1.07	1.60	64.48	813.1

[1] Treatment at × 20 concentration, then diluted.
[2] Treatment at × 5 concentration, then diluted.
[3] Filtered immediately after treatment.
[4] Sucrose and salts treated separately.
[5] Autoclaved 1 hour.
[6] Added 0.5 cc $(NH_4)_2S$ per liter.
[7] Substituted 0.64 g of K_2HPO_4.
[8] Substituted 0.52 g of K_3PO_4

Attempts to obtain an improvement by the use of filter pulp and excess iron (5 mg Fe per liter) were futile and gave the same results as those tabulated. The additional adsorptive capacity of the filter pulp and of the precipitate of iron salt seemed unnecessary. Another experiment with a solution which on mixing had a pH value of 7.19, and was filtered to remove the very slight precipitate formed, gave growth ratios of 16.91, 4.64, 1.09, and 1.20 for iron, zinc, copper, and manganese, respectively. These ratios, while equal to or superior to those obtained with the untreated solution, are inferior to those obtained with the treated solution, an indication that the adsorptive capacities of the precipitates formed during purification play a necessary part in the process.

Also of interest are the results obtained on the sporulation of the fungus and the accompanying variations in acidity of the nutrient solution resulting from the growth of the organism. This information has been tabulated in table 2. It will be noted that sporulation is inhibited if any of the four heavy metals under discussion are omitted from the nutrient solution. In the absence of copper, moreover, the deposition of pigment in the spore walls is interfered with and the spores are brown, yellow, or white, apparently according to the extent of the deficiency of this metal in the nutrient solution.

TABLE 2.—*Sporulation of Aspergillus niger and pH of nutrient solution at harvest with calcium carbonate employed as purificant*

Culture no.	Sporulation [1] in absence of indicated heavy metals						pH at harvest in absence of indicated heavy metals					
	All	Fe	Zn	Cu	Mn	None	All	Fe	Zn	Cu	Mn	None
1	1, bl	2, bl	1, bl	2, br	3, bl	4, bl	3.75	3.44	3.46	1.57	1.56	1.56
2	2, bl	3, bl	4, bl	4, br	0	5, bl	2.75	1.91	1.91	2.21	1.77	1.91
3	1, bl	1, bl	2, bl	2, y	3, br	5, bl	3.01	3.02	2.90	2.24	1.63	2.64
4	1, bl	2, bl	3, bl	4, br	4, bl	5, bl	3.20	2.98	2.87	1.89	1.85	2.10
5	1, bl	2, bl	2, bl	3, br	4, bl	5, bl	3.22	3.02	2.85	1.94	1.91	2.11
6	1, bl	3, bl	3, bl	4, br	5, bl	5, bl	2.97	2.77	2.77	1.78	1.79	1.93
7	2, bl	2, bl	3, bl	3, br	3, bl	4, bl						
8	2, bl	2, bl	3, bl	3, br	5, bl	5, bl	3.20	3.01	2.85	1.83	1.85	1.93
9	2, bl	2, bl	3, bl	3, br	5, bl	5, bl	3.20	2.89	2.88	1.96	1.85	2.01
10	1, j	1, j	1, j	2, y	0	5, j	2.95	2.89	2.98	1.78	1.53	1.72
11	1, j	1, j	2, j	2, y	0	3, bl	3.01	2.91	2.93	1.79	1.52	1.65
12	1, bl	1, bl	2, bl	2, y	2, bl	4, bl	3.19	2.99	2.89	2.08	1.58	1.91
13	1, bl	2, bl	5, bl	3, y	0	5, bl	3.35	3.02	2.65	2.54	1.50	2.44
14	1, j	1, j	2, j	2, w	0	4, bl	3.07	2.73	2.76	2.10	1.50	2.01
15	1, bl	1, bl	3, bl	3, br	2, bl	3, bl	3.29	3.07	2.80	1.50	1.46	1.37
16	1, bl	1, bl	2, bl	3, y	3, bl	4, bl	3.21	3.01	3.06	1.94	1.64	
17	1, bl	2, bl	2, bl	3, br	3, bl	4, bl	2.97	3.01	3.01	2.28	1.74	2.28
18	1, bl	1, bl	1, bl	2, y	2, bl	4, br	3.15	3.05	3.08	1.94	1.60	1.84
19	1, bl	1, bl	1, bl	2, br	2, br	4, bl	3.14	2.98	3.05	1.97	1.67	1.57
20	1, bl	1, bl	1, bl	2, br	1, bl	5, bl	3.04	2.91	2.91	2.04	1.67	1.57

[1] Amount of sporulation is indicated as 0 (sterile) to 5 (entirely covered with spores) and spore color by the initial letter or first 2 letters of the words jet, black, brown, tan, yellow, and white.

The presence in the nutrient solution of both iron and zinc seems to be necessary for the acid metabolism of the organism, whereas the addition of manganese and perhaps copper seems unnecessary. Manganese particularly does not seem to be required for acid formation and it is surmised that it bears some relation to the processes whereby the organic acids are decomposed. Since both copper and manganese are still present in the cultures in amounts sufficient for 50 to 75 percent of maximum growth, definite conclusions as yet cannot be drawn.

NUTRIENT-SOLUTION PURIFICATION WITH BASIC MAGNESIUM CARBONATE

The data of table 3 show the results obtained by the substitution of basic magnesium carbonate for calcium carbonate in freeing the nutrient solution from heavy metals. The results are similar to those obtained with calcium carbonate. However, the use of basic magnesium carbonate possesses certain advantages over that of calcium carbonate. It avoids the introduction of a nonnutrient, and heat treatment may be continued for 20 minutes in the steamer at 100° C. instead of in the autoclave at 120.5°, since the basic magnesium carbonate reacts quickly. Furthermore, the nutrient solution so purified seems to be nearly free from calcium and shows no trace of this element when tested with potassium fluoride or ammonium oxalate. Moreover, the absence of calcium to the degree indicated in these tests does not cause a decrease in the maximum yield. The disadvantage of the method is that it is critical for concentration of purificant employed, an excess resulting in the more or less complete removal of phosphate.

TABLE 3.—*Effect of variations in purification method with basic magnesium carbonate upon growth of Aspergillus niger in a nutrient solution containing 5 percent sucrose* [1]

Culture no.	Content in nutrient solution,				Initial pH	Yield without heavy metals	Growth ratio with—					Maximum individual yield
	NH_4NO_3	KH_2PO_4	$MgSO_4.7H_2O$	$4MgCO_3.Mg(OH)_2.5H_2O$			Fe	Zn	Cu	Mn	All	
	Grams per liter	*Grams per liter*	*Grams per liter*	*Percent*		*Mg*						*Mg*
1	2.5	1.0	0.3	0.04	7.15	13.6	51.91	5.26	1.42	1.44	68.00	925.5
2	2.5	1.0	.3	.06	7.39	10.5	54.17	6.10	1.58	1.56	99.57	1,057.7
3	2.5	1.0	.3	.07	7.52	22.7	31.43	28.06	1.53	1.89	41.53	984.2
4	2.5	1.0	.3	.08	7.62	23.2	53.10	5.86	1.56	1.82	36.39	905.8
5 [2]	2.5	1.0	.3	.07	8.15	11.1	11.55	20.20	.82	1.03	31.12	525.2
6	2.5	1.0	1.0	.10	7.60	7.7	52.46	7.32	1.61	1.14	16.35	196.4
7	5.0	1.0	1.0	.10	7.29	12.6	45.26	15.19	1.96	1.48	36.28	479.3
8	2.5	2.0	1.0	.10	6.94	12.5	33.26	4.97	1.49	1.46	76.37	991.6

[1] Duration of treatment was 24 hours in all cases. [2] Added 0.5 cc $(NH_4)_2S$ per liter.

The data on sporulation and acidity at harvest, obtained in the experiments tabulated in table 3, have been summarized in table 4. The results are essentially the same as those given with the calcium carbonate method. The effects of omission of heavy metals on sporulation seem to be intensified, however, while the variations in acidity remain about the same.

TABLE 4.—*Sporulation of Aspergillus niger and pH of nutrient solution at harvest with basic magnesium carbonate employed as purificant*

Culture no.	Sporulation in absence of indicated heavy metals [1]						pH at harvest in absence of indicated heavy metals					
	All	Fe	Zn	Cu	Mn	None	All	Fe	Zn	Cu	Mn	None
1	1, bl	2, bl	4, bl	3, y	1, bl	4, bl	2.96	2.93	2.37	1.95	1.71	1.73
2	1, bl	2, bl	4, bl	3, y	1, bl	4, bl	3.27	3.03	2.42	2.22	1.68	2.22
3	1, bl	1, bl	2, bl	2, y	2, bl	4, bl	3.28	3.00	3.04	1.83	1.63	1.78
4	1, bl	1, bl	4, bl	2, br	1, bl	3, bl	3.14	3.05	2.44	1.81	1.64	1.81
5	1, bl	1, bl	1, bl	1, bl	0	2, bl	2.29	3.08	3.16	1.85	1.74	1.81
6	0	0	2, bl	2, y	2, bl	3, bl	3.80	6.88	3.35	2.50	2.23	2.26
7	0	1, bl	2, bl	1, br	1, bl	3, bl	3.84	3.24	2.93	1.99	2.25	1.95
8	0	2, bl	5, bl	2, br	1, bl	5, bl	3.08	3.05	2.49	2.02	1.69	2.02

[1] See footnote 1, table 2.

NUTRIENT-SOLUTION PURIFICATION WITH ADSORBENT CHARCOAL

The experimental work with charcoal as purificant given in table 5 was planned primarily to ascertain whether the use of adsorbent charcoal in conjunction with a purificant of definite chemical composition would lead to any improvement. Basic magnesium carbonate was selected instead of calcium carbonate because the lower initial acidities brought about through its use are nearer the acidity

(pH 8) recommended for utilization of charcoal. Other compounds of the alkaline earth metals, such as the oxides, hydroxides, or tribasic phosphates, probably would operate similarly. One experiment with calcium carbonate also has been included for comparison. The charcoals compared are (A) Carbex E (carboraffin), (B) cane-sugar charcoal, (C) decolorizing charcoal, and (D) medicinal charcoal purified by acid. None of these charcoals was free from ash constituents; even the cane-sugar charcoal contained 2.07 percent of ash.

The results obtained by supplementing the action of basic magnesium carbonate with adsorbent charcoal do not differ in any important degree from those previously discussed. All indications point to the conclusion that the use of charcoal is advisable only if it is necessary to avoid the use of an alkaline earth carbonate, hydroxide, or oxide. The lack of uniformity in results with different charcoals is most apparent in the acidities at harvest. These variations of course are not unexpected, inasmuch as organic compounds and 2 to 5 percent of ash constituents are usually present in an adsorbent charcoal. Miller (*6*) asserts that ash-free charcoal is not an effective adsorbent for cations no matter what its source, and claims that adsorption of cations is largely a property of the contaminants present in the carbon.

EFFECT OF SPORE EXTRACTION UPON SUBSEQUENT GROWTH OF ASPERGILLUS NIGER

The inability to obtain growth ratios higher than 2 (50 percent of maximum yield) with copper and manganese in these investigations must be due to a technic inadequate to remove these metals entirely from the nutrient solution, to their introduction as contaminants with the heavy metals intentionally added, to the presence of these materials in the spores, or to a combination of these causes. The only alternative is the assumption that an element may be essential for a physiological function and yet not be of the first importance to growth. This alternative would seem improbable, however, inasmuch as other data on hand show that the absence of any of the elements essential to *Aspergillus niger* results in the almost complete suppression of growth. Hence it seemed advisable to attempt to determine experimentally the extent to which the growth of the fungus is influenced by the presence of copper and manganese in the spores. Parallel studies also were made on the effect of iron and zinc on growth.

Extraction of the spores with dilute alkali was adopted initially, since the black pigment (aspergillin) in the spores is known to contain iron and to be soluble in dilute alkalies. The possibility that the storage of copper and manganese in the spores is associated with that of iron rendered this procedure attractive for a first trial. Tests demonstrated that the pigment could be extracted from the spores with potassium bicarbonate (0.5 to 30.0 g per liter) without injury to the spores. Experiments in which ammonium hydroxide was employed also were performed. The results are tabulated in table 6. The increased growth ratios with iron, copper, and manganese would indicate the presence of these materials in the spores and the feasibility of at least their partial removal. The negative results with zinc are to be attributed to its presence in the potassium bicarbonate and ammonium hydroxide employed, since the use of redistilled ammonia led to a higher growth ratio with zinc.

TABLE 5.—*Development of Aspergillus niger in nutrient solutions purified with calcium carbonate or basic magnesium carbonate and with various adsorbent charcoals* [1] *employed as supplementary purificants*

Heavy metals omitted	0.1 percent $CaCO_3$ and 0.1 percent (A)			0.07 percent $4MgCO_3.Mg(OH)_2.5H_2O$ and indicated percentages of various charcoals															
				0.1 percent (A)			0.5 percent (A)			0.5 percent (B)			0.5 percent (C)			0.5 percent (D)		
	Growth ratio	pH at harvest	Sporulation	Growth ratio	pH at harvest	Sporulation	Growth ratio	pH at harvest	Sporulation	Growth ratio	pH at harvest	Sporulation	Growth ratio	pH at harvest	Sporulation	Growth ratio	pH at harvest	Sporulation
	[2] 17.0	[3] 5.80	.....	[2] 8.3	[3] 7.37	.....	[2] 13.3	[3] 7.34	.....	[2] 15.5	[3] 7.59	.....	[2] 9.6	[3] 7.71	.....	[2] 22.6	[3] 7.23	.....
All	64.36	3.35	1, bl	118.78	3.36	1, bl	82.70	3.32	1, bl	66.54	3.23	1, j	33.65	3.66	1, j	45.51	3.01	2, j
Fe	51.13	2.95	1, bl	39.28	2.99	1, bl	52.38	3.09	1, bl	47.31	3.15	1, j	29.91	3.84	1, j	43.03	3.09	2, j
Zn	20.96	2.88	3, bl	64.02	3.06	1, bl	28.04	2.90	2, j	30.88	3.11	2, j	20.44	3.39	2, j	30.43	3.02	3, j
Cu	1.04	2.38	3, br	1.21	2.16	2, br	1.10	3.29	3, t	1.08	3.06	3, t	.52	1.98	2, w	1.11	3.09	4, t
Mn	1.02	2.44	4, bl	1.22	1.85	2, bl	.97	2.65	4, b	1.54	1.65	0	1.59	2.04	2, j	1.26	1.82	1, j
None	[4] 1,100.0	2.46	4, bl	[4] 1,124.8	3.05	3, bl	[4] 1,181.7	2.79	4, bl	[4] 1,053.9	3.58	4, bl	[4] 661.1	1.85	2, j	[4] 1,061.8	3.22	5, bl

[1] Letters indicate the different charcoals as follows: A, Carbex E (carboraffin); B, cane-sugar charcoal; C, decolorizing charcoal; D, medical charcoal purified by acid.
[2] Average yield in milligrams without heavy metals.
[3] Initial pH.
[4] Maximum individual yield in milligrams.

TABLE 6.—*Effects of spore extraction on the development of Aspergillus niger in a 2.5–1.0–1.0 nutrient solution purified with 0.1 percent of calcium carbonate*

Heavy metals omitted	Spores extracted with $KHCO_3$ of indicated concentration						Spores extracted with NH_4OH of indicated concentration											
	0.5 percent			3.0 percent			1.0 percent			5.0 percent			10.0 percent			7.0 percent [4]		
	Growth ratio	pH at harvest	Sporulation	Growth ratio	pH at harvest	Sporulation	Growth ratio	pH at harvest	Sporulation	Growth ratio	pH at harvest	Sporulation	Growth ratio	pH at harvest	Sporulation	Growth ratio	pH at harvest	Sporulation
	[1] 23.0	[2] 6.77	.....	[1] 15.1	[2] 6.67	.....	[1] 18.8	[2] 5.98	.....	[1] 6.9	[2] 5.98	.....	[1] 14.5	[2] 6.65	.....	[1] 12.7	[2] 6.53	.....
All	41.06	2.99	1, bl	64.97	3.08	1, bl	49.13	3.15	1, j	146.61	3.34	1, j	45.12	3.21	2, j	65.32	3.15	1, j
Fe	39.84	2.99	2, bl	112.77	3.05	1, bl	46.18	2.85	1, j	83.60	3.27	1, j	30.71	3.06	2, j	45.83	3.07	1, j
Zn	20.66	2.79	2, bl	5.26	2.41	4, bl	7.87	2.39	2, j	11.83	2.47	2, j	28.44	3.12	2, j	33.18	3.01	1, j
Cu	1.99	1.81	3, bl	1.67	2.10	2, y	1.76	1.80	1, w	2.84	1.77	1, y	1.09	1.74	2, br	1.19	2.01	2, t
Mn	1.09	1.88	4, bl	1.38	1.74	2, bl	2.24	1.60	1, j	2.28	1.60	1, j	1.97	1.63	2, j	1.58	1.68	2, j
None	[3] 1,040.3	2.01	5, bl	[3] 982.5	1.84	4, bl	[3] 923.6	1.92	3, j	[3] 1,011.6	1.78	3, j	[3] 654.2	1.65	2, j	[3] 829.5	1.85	3, j

[1] Average yield in milligrams without heavy metals.
[2] Initial pH.
[3] Maximum individual yield in milligrams.
[4] Purified by distillation.

DISCUSSION

To judge from the description by Britton (*2*) of the factory methods for the purification of cane sugar and the acidity at which the heavy metals are precipitated as phosphates, it would seem that the method of nutrient purification with calcium carbonate is quite similar to the factory procedure for the purification of sucrose. The heavy-metal impurities would appear to be precipitated largely as phosphates, though some may also separate out as hydroxide, carbonate, or basic carbonate, depending on the acidity of precipitation. On the assumption that a precipitate of the most insoluble compound is always formed, it is probable that under these conditions practically all the heavy metals will undergo precipitation in one form or another. Thus, when basic magnesium carbonate is used as the purificant even barium, strontium, and calcium probably will be removed if the proper quantity of purificant (and therefore the appropriate acidity) is utilized, since the salts of the latter elements are precipitated at a higher acidity than is magnesium. In principle the method seems dependent upon increasing the alkalinity of the solution in the presence of an alkaline earth sufficiently to cause the precipitation of the alkaline earth simultaneously with that of the heavy metal it is desired to remove. The precipitate of the alkaline earth compound thus serves as a "gatherer" or adsorbent. The chemistry of the process therefore is elementary. As suggested by Hopkins (*5*), perhaps also other cations, such as some of the heavy metals, may be found to answer the same purpose. It is immaterial whether the alkaline earth be added before or after the decrease in acidity, and the means whereby the decrease in acidity is brought about also is of no consequence. Neither is it important whether the purificant is added as an integral part of the nutrient solution.

The speed of reaction with calcium carbonate seems to be slower, and it is stated by Hillebrand and Lundell (*4*) that the limit of alkalinity corresponds to pH 7.4, whereas the basic magnesium carbonate reacts rapidly and can produce pH 9.5 if sufficient be added. With the former material the reaction never goes to completion under the conditions employed, while with the basic magnesium carbonate the reaction is always complete.

Since the precipitates formed during purification are never absolutely insoluble it cannot be claimed that the process completely removes the heavy metals. The efficiency of removal differs with the element and appears to be greater with iron and zinc than with copper or manganese. A puzzling feature in this connection is that whereas formerly the purified solution was always light yellow as the result of caramelization (*8*) the solutions in the present series of experiments were always colorless.

The results obtained through extraction of the spores of *Aspergillus niger* are distinctly encouraging and would seem to indicate the utility of this procedure as an experimental tool to accentuate heavy-metal deficiencies with the fungi. This procedure may prove feasible also with seed of green plants and appropriately selected compounds. The quantities of heavy metal stored in the spore or seed in some cases may be found sufficient in quantity to provide completely for the growth of the organism. This does not seem to be the case with *A. niger*, however.

Data obtained in an unsuccessful attempt to remove all halogens from the nutrient solution are illustrative of the magnitudes dealt with in these experiments and of the sensitivity of response of the organism. The addition of silver nitrate (5 mg Ag per liter) to the nutrient solution, followed by treatment with calcium carbonate to remove excess silver, gave upon filtration a solution presumably free of halogens. The presence of silver in the solution was revealed, however, through absence of growth upon inoculation. Nevertheless, a series of simultaneous cultures to which slight excesses of sodium chloride, sodium bromide, and sodium iodide had been added gave characteristic precipitates and yields of 0, 121.3, and 748.2 mg per culture, respectively. The solubilities of silver chloride, silver bromide, and silver iodide in water are 1.5, 0.11, and 0.003 mg per liter, respectively. On the assumption of equal solubility of silver iodide in water and in 5-percent sucrose, the results would indicate that 1.5 parts per billion of silver ion are sufficient to cause a loss of about 25 percent in yield. Actual determinations later gave decreases in yield of 9.9 and 6.4 percent with 1 part per billion of silver ion, though in unpurified solutions.

Without attempting to minimize the value of the method of nutrient-solution purification for biological study, nevertheless it must be admitted to be a makeshift and of value mainly because of the presence of excessive quantities of impurities in the commercial chemicals available for work of this character. Its disadvantage consists in the inability to predict the exact composition of a nutrient solution after treatment and consequently the limitations in its employment for critical study of nutrient proportions. It is estimated that a twentyfold improvement in the purity of commercial chemicals would permit one to dispense with the method of nutrient-solution purification in nutrition studies with plants. To be entirely satisfactory an increase of at least fiftyfold would be required in the purity of the compounds employed.

Pending the development of methods for producing chemicals that might be referred to as biologically pure and of adequate chemical or spectroscopic tests for ascertaining their purity, the calcium carbonate method of nutrient purification seems to be the best procedure available for the removal of the last traces of heavy metals. This method, or variants of it, has already been applied successfully to determine the heavy-metal requirements of various fungi, of algae, and of *Lemna*. Since the salts may be subjected to purification successfully in the absence of sucrose (table 1, culture no. 11) there is no reason to think that the method cannot be applied to the study of the heavy-metal requirements of the higher plants. Moreover, the agreement in results obtained with selected commercial chemicals and with the various modifications of the purification method here discussed makes it evident that these results on the requirements of *Aspergillus niger* for iron, zinc, copper, and manganese cannot be accidental and should serve definitely to establish these needs. These heavy metals have been claimed to be essential also to green plants and to animals. Results with the method of nutrient-solution purification obtained with quartz apparatus were essentially similar to those obtained with pyrex glassware.

SUMMARY

Purification of the nutrient solution for the removal of heavy metals may be accomplished by treating the nutrient solution with calcium carbonate and filtering while hot. The method is not critical and gives good results with iron, zinc, copper, and manganese under a wide variety of conditions and procedures. The removal of heavy metals appears to be dependent on their coprecipitation with an alkaline earth as phosphate, carbonate, or hydroxide by a decrease in acidity. The use of adsorbent charcoal to supplement the action of an alkaline-earth purificant is unnecessary and causes a decrease in experimental precision. Extraction of the spores of *Aspergillus niger* with alkaline solutions effects partial removal of iron, copper, and manganese stored in the spores in preparation for subsequent growth, and so leads to an accentuation of deficiency effects with these metals.

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A SIMPLE, ACCURATE METHOD OF COMPUTING BASAL AREA OF FOREST STANDS[1]

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INTRODUCTION

Basal area is a measure of a timber stand by the use of which the errors peculiar to measurement by volume are avoided, and by which a more accurate picture of the timber is provided than by diameter measurement alone. For these reasons it is widely used. The statistics of basal area which are commonly sought for various purposes are: The sum; the mean; the diameter of mean basal area; the standard deviation; the product moment, or sum of cross products; the growth of a tree; the growth of a stand or plot; the mean growth; and the standard deviation of growth. The common methods of obtaining these statistics are either exceedingly laborious or introduce systematic errors. The purpose of this paper is to present a method which is both precise and easy to apply.

REVIEW OF METHODS

The usual way of determining basal area of a timber stand is to list the basal area of every measured tree, using a basal-area table,[2] and compute the desired statistics from this list. Where there are many trees, this method is slow and laborious, but accurate in principle. In practice it lacks a systematic check, and errors creep in which may go unnoticed or may at best be troublesome to correct. With this method, the computation of any statistics besides the mean and sum of basal area and diameter of average basal area is not usually worth the effort.

Another common procedure in computing statistics of basal area is to group the measured diameters into classes and select a value for each class in using the basal-area table. This is obviously much less tedious than the other method and is also less liable to troublesome numerical mistakes. However, it introduces a systematic error due to grouping, the magnitude and direction of which depends on the diameter distribution and the class value chosen. Another class of error is introduced when the usual interpolation in the table is made by proportional parts; this error is always negative in sign.

THE FORMULA METHOD

The proposed method is derived from the analytical definition of basal area—

$$b = \frac{\pi d^2}{(4)(144)} \qquad (1)$$

[1] Received for publication May 3, 1935; issued December, 1935.

[2] For simplicity, diameters are assumed to be perfect circles and without errors as measured.

where b is basal area in square feet and d is diameter in inches.

If $$k=\frac{\pi}{(4)(144)}=0.005454\ldots.$$

this may be expressed: $$b=kd^2 \qquad (2)$$

Using this definition and the formulae which follow, computation of any of the above statistics of basal area is both easy and precise. This may be called the "formula method", to contrast with the usual table method.

APPLICATION TO UNGROUPED DATA

Computations of basal area by the formula method may be made with or without grouping. Ungrouped data generally mean a small sample, and with few trees and only simple statistics desired, the quickest procedure is to use the basal-area table. As the job increases in complexity the formula method becomes increasingly advantageous.

It readily follows from equation (2) that total basal area—

$$\Sigma b=k\Sigma d^2, \qquad (3)$$

and that mean basal area—

$$\frac{\Sigma b}{N}=\frac{k}{N}\Sigma d^2 \qquad (4)$$

where Σb is the sum and $\frac{\Sigma b}{N}$ mean of basal area, and Σd^2 is the sum of the squared diameters. The diameter of average basal area is from (2) and (4) above, $d'=\sqrt{\frac{\frac{\Sigma b}{N}}{k}}=$

$$\sqrt{\frac{\frac{k\Sigma d^2}{N}}{k}}=\sqrt{\frac{\Sigma d^2}{N}} \qquad (5)$$

Standard deviation of basal area [3]

$$\sigma_b=\sqrt{\frac{\Sigma\left(b-\frac{\Sigma b}{N}\right)^2}{N}}=\sqrt{\frac{\Sigma b^2}{N}-\left(\frac{\Sigma b}{N}\right)^2}=$$

$$\frac{1}{N}\sqrt{N\Sigma b^2-(\Sigma b)^2}=\frac{k}{N}\sqrt{N\Sigma d^4-(\Sigma d^2)^2} \qquad (6)$$

In small samples the standard deviation must be multiplied by

$$\sqrt{\frac{N}{N-1}}$$

[3] An approximate standard deviation of basal area may be obtained when the diameter distribution is nearly normal, which avoids Σd^4, as follows: From the moments of the normal curve, $\frac{\Sigma(x-M)^r}{N}$, it may be shown that $\Sigma d^2=N(\sigma^2+M^2)$ and $\Sigma d^4=N(3\sigma^4+6M^2\sigma^2+M^4)$; substituting these values in equation (6)

$$\frac{k}{N}\sqrt{N\Sigma d^4-(\Sigma d^2)^2}=$$

$$k\sqrt{2\sigma^4+4M^2\sigma^2}=k\sqrt{2\sigma^2(\sigma^2+2M^2)}=$$

$$k\sqrt{2\left[\frac{\Sigma d^2}{N}-\left(\frac{\Sigma d}{N}\right)^2\right]\left[\frac{\Sigma d^2}{N}-\left(\frac{\Sigma d}{N}\right)^2+2\left(\frac{\Sigma d}{N}\right)^2\right]}=$$

$$k\sqrt{2\sqrt{\left(\frac{\Sigma d^2}{N}\right)^2-\left(\frac{\Sigma d}{N}\right)^4}} \qquad (7)$$

This approximation is useful because it uses values that are easily obtained, and even though in error it may serve to show that it is unnecessary to compute the true standard deviation.

A simple way to obtain the sums of the higher powers of d required in the above formulae is to list diameters by size in descending order and tabulate diameter, number, the product of these two, and three cumulative sums as follows:

d	n	nd	C_1	C_2	C_3
m	n_m	mn_m	mn_m	mn_m	mn_m
$m-1$	n_{m-1}	$(m-1)n_{m-1}$	mn_m $+(m-1)n_{m-1}$	$2mn_m$ $+(m-1)n_{m-1}$	$3mn_m+$ . . .
$m-2$	n_{m-2}	$(m-2)n_{m-2}$	mn_m $+(m-1)n_{m-1}$ $+(m-2)n_{m-2}$	$3mn_m$ $+2(m-1)n_{m-1}$ $+(m-2)n_{m-2}$	$6mn_m+$ . . .
3	n_3	$3n_3$			
2	n_2	$2n_2$			
1	n_1	n_1	S_0	S_1	S_2
Total	N	S_0	S_1	S_2	S_3

Then $\Sigma d^2=S_1$ and $\Sigma d^4=6S_3-6S_2+S_1$.

These cumulative sums require that d be continuous from m to 1 inclusive, n_i may be zero, but its row will have entries in C_1, C_2, and C_3. Note that this procedure provides a check, in that the last entry under the cumulative sums is the sum of the preceding column.

The product moment, or sum of cross products, is a statistic used in correlation analysis and regression equations. Where x is an associated variable—

$$\Sigma bx=k\Sigma d^2x \tag{8}$$

Σbx may be readily obtained either by direct multiplying or more simply by the method of cumulative sums shown above. The sort is still on d, but, instead of showing nd, show Σx for each d value; then $\Sigma x=S_0$ and $\Sigma d^2x=2S_2-S_1$.

The growth of a single tree in basal area is rarely needed, but its derivation aids in understanding what follows. Let Δd be growth in diameter and Δb be growth in basal area, then d and b are initial diameter and basal area, and $d+\Delta d$ and $b+\Delta b$ are diameter and basal area at any subsequent period.

Now— $$b=kd^2$$

$$b+\Delta b=k(d+\Delta d)^2=k(d^2+2d\Delta d+\Delta d^2)$$

and by subtraction—

$$\Delta b=k(2d\Delta d+\Delta d^2) \tag{9}$$

From which it readily follows that total basal-area growth,

$$\Sigma\Delta b = k(2\Sigma d\Delta d + \Sigma\Delta d^2) \tag{10}$$

and mean growth in basal area

$$\frac{\Sigma\Delta b}{N} = \frac{k}{N}(2\Sigma d\Delta d + \Sigma\Delta d^2) \tag{11}$$

There appears, unfortunately, to be no simple, direct means of obtaining σ_{Δ_b} from d and Δd, but it may be computed in the usual way—

$$\sigma_{\Delta_b} = \sqrt{\frac{\Sigma\Delta b^2}{N} - \left(\frac{\Sigma\Delta b}{N}\right)^2} \tag{12}$$

or with the usual correction factor for small samples. This formula requires the squaring, summing, and averaging of each individual Δb to obtain the first term, while the second may be secured from formula (11) above.

APPLICATION TO GROUPED DATA

With grouped data the practice usually recommended is to use the mid point of each class as the class value.[4] Sheppard[5] has shown that the average effect of grouping, using the mid point as the class value, is to introduce a positive bias of one-twelfth the square of the class interval in the average square when the class interval is constant, the distribution is continuous, and the class frequencies decrease very slowly in the tails (i. e., the frequency curve makes contact of high order with the abscissa). Since total or average basal area is a function of the sum of squares of diameter, it follows that with grouped data a correction of $\frac{nkh^2}{12}$ or $\frac{kh^2}{12}$, when h is the class interval, should be made. In the mean, where 1-inch classes are used, this amounts to only 0.0004545 . . . and it is of course correspondingly small in the total. It is evident that for all common purposes this correction is too small to be of any practical value. However, it should be noted that Sheppard's correction is meant to apply to the average result of grouping. In practice it is never worth while to obtain this average; but for certain distributions the results of a particular grouping may be as much as 10 percent different from values obtained without grouping. In general, grouped data will rarely produce the same result as would be obtained without grouping, but the discrepancies will be small and the grouping alone causes only a slight bias, if any. There is some loss in precision, but the statistics are much easier to compute.

But basal area as customarily obtained by grouping suffers a greater loss of precision than is necessary. The usual grouping of

[4] It might be supposed that the arithmetic class mean would provide a satisfactory class value, but it may be readily shown that this is too small by k times the sum of squares within classes.

[5] SHEPPARD, W. F. ON THE CALCULATION OF THE MOST PROBABLE VALUES OF FREQUENCY-CONSTANTS FOR DATA ARRANGED ACCORDING TO EQUIDISTANT DIVISIONS OF A SCALE. London Math. Soc. Proc. (1897-98) 29:353-380. 1898.

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diameters puts the mid point of 1-inch classes at 0.05 greater than the even inch, and few basal-area tables show classes with diameter intervals of less than one-tenth of an inch. It thus becomes necessary to interpolate, and this is usually effected by using proportional parts. The error so introduced is small for individual trees, but it is always negative and may become appreciable in a sum.

Errors are also made by using the even inch as the mid point of the class, though it is usually 0.05 inch too small. This error may be readily corrected as shown below, but the correction is rarely made. Let A be the mid point of a class and a any deviation from A:

$$\Sigma b = k\Sigma d^2 = k\left(\Sigma A^2 - \frac{Nh^2}{12}\right)$$

$k\Sigma A^2$ is obtained by using the true mid point.
$k\Sigma(A+a)^2$ is obtained by using any other point.
Ignoring k for the moment—

$$\Sigma(A+a)^2 = \Sigma A^2 + 2a\Sigma A + Na^2$$

whence

$$\Sigma A^2 = \Sigma(A+a)^2 - 2a\Sigma A - Na^2 = \Sigma(A+a)^2 - 2a\Sigma(A+a) + Na^2.$$

From this it is evident that no injury is done by using any arbitrary value desired for the class value in obtaining the sum of squares if the proper corrections are made. As a matter of fact, for ease of computation where the true mid point is some relatively awkward number, such as 5.05, the method of the assumed mid point is much simpler. Furthermore, since $\Sigma A^r = \Sigma(A+a-a)^r$, it is very simple to obtain any of the higher moments needed even though a false class value is used:

$$\Sigma A = \Sigma(A+a) - Na \tag{13}$$

$$\Sigma A^2 = \Sigma(A+a)^2 - 2a\Sigma(A+a) + Na^2 \tag{14}$$

$$\Sigma A^3 = \Sigma(A+a)^3 - 3a\Sigma(A+a)^2 + 3a^2\Sigma(A+a) - Na^3 \tag{15}$$

$$\Sigma A^4 = \Sigma(A+a)^4 - 4a\Sigma(A+a)^3 + 6a^2\Sigma(A+a)^2 - 4a^3\Sigma(A+a) + Na^4 \tag{16}$$

etc.

The proposed formula method uses formulae (13) to (16), with grouped data, to obtain ΣA^r.

$$\Sigma A^r = \Sigma d^r + \text{Sheppard's correction.}$$

The formulae of ungrouped data, (3) to (12) inclusive, may then be used to obtain whatever statistics of basal area are needed.

A NUMERICAL EXAMPLE

The following example presents a summary of field data and the computations essential to the use of the preceding formulae.

From the original measurements (which are here excluded for the sake of brevity)—

$$N = 165$$
$$\Sigma d = 548.0$$
$$\Sigma d^2 = 1966.64$$
$$\Sigma d^4 = 31532.4704$$
$$\Sigma b = 10.724 \text{ (from table of basal area)}$$
$$k\,\Sigma d^2 = 10.726.$$

Table 1 shows the data grouped in the conventional manner with computations for two estimated values for each class.

TABLE 1.—*Computation of the sum of basal area by usual methods*

Diameter breast high interval (inches)	Trees (n)	Mid-point (A)	Approximate mid point ($A+a$)	Basal area by—			
				True value	A	$A+a$	Individual trees
	Number	*Inches*	*Inches*	*Sq. ft.*	*Sq. ft.*	*Sq. ft.*	*Sq. ft.*
1.6–2.5	43	2.05	2	1.2244	0.989	0.946	1.216
2.6–3.5	66	3.05	3	3.4368	3.300	3.234	3.440
3.6–4.5	37	4.05	4	3.2952	3.293	3.219	3.700
4.6–5.5	17	5.05	5	2.3568	2.363	2.312	2.356
5.6–6.5	2	6.05	6	7.4128	7.398	7.392	7.412
Total	165			10.726	10.343	10.103	10.724

Table 2 shows the data grouped and computed by the formula method.

TABLE 2.—*Illustration of the formula method in obtaining the sum of basal areas*

Approximate mid point of diameter breast high ($A+a$) (inches)	Trees (n)	n ($A+a$)	C_1	C_2	Approximate mid point of diameter breast high ($A+a$) (inches)	Trees (n)	n ($A+a$)	C_1	C_2
	Number					*Number*			
6	2	12	12	12	2	43	86	529	1,326
5	17	85	97	109	1			529	1,855
4	37	148	245	354					
3	66	198	443	797	Total	165	529	1,855	

Σd^2 is approximately $\Sigma(A+a)^2-2a\ \Sigma(A+a)+Na^2$ or $1855-2(-0.05)(529)+(165)(-0.05)^2=1908.3125$. $\Sigma b=k\Sigma d^2$ or approximately $1908.3125\ k=10.408$. Note that Σb without correction (14) is $1855k$ or 10.117.

Of the six values for the sum of basal area, the one most likely to be right is 10.726 obtained from $k\Sigma d^2$. That obtained by summing the tabular values for individual trees checks as closely as can be expected.

The result of using the true mid points is contrary to what would be expected for this method on the average, but it should be remembered that this is a particular sample and the generalization applies to the averages of all possible groupings. This does not imply or even suggest that there is anything wrong with the sample, as certain other unexpected results might. In this particular example the 10 possible groupings of diameter were computed and total basal area varied from 9.520 to 12.255 and averaged 11.039. Sheppard's correction of −0.075 helped, but at 10.964 is still 2 percent too large.

The employment of the even inch as the class mid point yields, as was expected, a result that is too small. When the correction 53.3125 is added to 1,855 it precisely checks 1,908.3215, as it should.

The discrepancies in total basal area as obtained from the table and from summing the squares and correcting by the factor k are due to the lack of precision of the table, as is shown by the following comparison (table 3).

TABLE 3.—*Comparison of errors introduced by using too few decimal places*

A (inches)	Table	kd^2	$A+a$ (inches)	Table	kd^2
	Sq. ft.	*Sq. ft.*		*Sq. ft.*	*Sq. ft.*
2.05	0.023	0.02292	2	0.022	0.02182
3.05	.050	.05074	3	.049	.04909
4.05	.089	.08946	4	.087	.08726
5.05	.139	.13909	5	.136	.13635
6.05	.199	.19963	6	.196	.19634

These differences, although small for a particular entry, become increasingly important as the n of the class increases and even without a tendency to be conservative introduce a bias toward too small a value when using the basal-area table with any sort of class values. Note that 4 of the 5 entries in each group have a larger value for kd^2 than for the table value. (Interpolation for the values of A was made assuming a straight line, which is the common practice, although it is admittedly erroneous.) It thus becomes apparent that to obtain precision in the sum of basal area by means of grouped data equal to that obtained from ungrouped data using a table which shows diameter to 1 decimal place and basal area to 3 decimal places, it is essential to have a table showing diameter to 2 and basal area to 5 decimal places, as well as to correct for the errors of grouping, or to use other means than a table.

The other statistics of basal area as computed by the formulae are presented in table 4:

TABLE 4.—*Comparison of other statistics of basal area as ascertained by various methods*

Source	Average basal area	Diameter breast high of average basal area	$\sqrt{\frac{\Sigma d^2}{N}}$	Standard deviation σ_b	Approximate standard deviation σ'_b
	Sq. ft.	*Inches*	*Inches*	*Sq. ft.*	*Sq. ft.*
Table and A	0.0627	3.4			
Table and $A+a$	.0612	3.3			
Individual d	.0650	3.45	3.45	0.03819	0.03483
Table and individual d	.0650	3.45			
Sum of A^2	.0631	3.4	3.40		.03565
Sum of $(A+a)^2$	.0613	3.3	3.35		.03512

The points of interest here are already discussed for sums and will differ only in their values, not in their relation to one another. The σ'_b obtained from any of the four methods shows essential agreement with σ_b, but there is a discrepancy in the wrong direction which is too large when there is any doubt of the result of the test for which it is used.

The formulae of cross products and growth, though equally important, are not illustrated because to do so would require another example and the arithmetic involves no new principles.

SUMMARY

Statistics of basal area may be readily obtained by the "formula method" without using a table of basal area. No one method will, however, meet all situations; the best way for each particular case depends on the precision required, the size of the sample, and the mechanical aids available. For very small samples, when only simple statistics such as the sum and mean are wanted, the basal-area table is the easiest way of obtaining them. For more complex statistics from relatively small samples, the most efficient procedure is to use formulae (3), (4), (5), (6), (8), (10), and (12), as needed, without grouping. For large samples, the easiest way is to group, using intervals of even inches, and make the corrections indicated by formulae (13) to (16), remembering that precision is always lost by grouping.

The formulae presented are reassembled here for ready reference:

Total basal area:

$$k\Sigma d^2 \tag{3}$$

Average basal area:

$$\frac{k}{N}\Sigma d^2 \tag{4}$$

Diameter of average basal area:

$$\sqrt{\frac{\Sigma d^2}{N}} \tag{5}$$

Standard deviation of basal area:

$$\frac{k}{N}\sqrt{N\Sigma d^4-(\Sigma d^2)^2} \tag{6}$$

or

$$\frac{k}{N}\sqrt{\frac{N}{N-1}}\sqrt{N\Sigma d^4-(\Sigma d^2)^2}$$

Approximate standard deviation of basal area:

$$k\sqrt{2}\sqrt{\left(\frac{\Sigma d^2}{N}\right)^2-\left(\frac{\Sigma d}{N}\right)^4} \tag{7}$$

or

$$k\sqrt{\frac{2N}{N-1}}\sqrt{\left(\frac{\Sigma d^2}{N}\right)^2-\left(\frac{\Sigma d}{N}\right)^4}$$

Product moment:

$$\Sigma bx=k\Sigma d^2x \tag{8}$$

Basal area growth:

$$\Delta b=k(2d\Delta d+\Delta d^2) \tag{9}$$

Total basal-area growth:

$$\Sigma\Delta b=k(2\Sigma d\Delta d+\Sigma\Delta d^2) \tag{10}$$

Standard deviation of basal area growth:

$$\sqrt{\frac{\Sigma\Delta b^2}{N}-\left(\frac{\Sigma\Delta b}{N}\right)^2} \tag{12}$$

or

$$\sqrt{\frac{N}{N-1}}\sqrt{\frac{\Sigma\Delta b^2}{N}-\left(\frac{\Sigma\Delta b}{N}\right)^2}$$

Corrections for using a false mid point:
Sum of first power:

$$Na \tag{13}$$

Sum of second power:

$$2a\Sigma(A+a)-Na^2 \tag{14}$$

Sum of third power:

$$3a\Sigma(A+a)^2-3a^2\Sigma(A+a)+Na^3 \tag{15}$$

Sum of fourth power:

$$4a\Sigma(A+a)^3-6a^2\Sigma(A+a)^2+4a^3\Sigma(A+a)-Na^4 \tag{16}$$

THE EFFECT OF ONE AND OF TWO SEEDLING LETHALS IN THE HETEROZYGOUS CONDITION ON BARLEY DEVELOPMENT[1]

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INTRODUCTION

The effect of recessive characters in the heterozygous condition may have some economic importance in yield of cereal crops. The studies reported in this paper were made to determine the effect of 1 or of 2 lethal factors in the heterozygous condition on the growth of barley plants.

LITERATURE REVIEW

A summary of the recent literature on the effect of lethal characters on the growth of various crops is given in a previous publication by Robertson.[2]

A previous paper reported that the factor pair $A_c a_c$ for white versus green seedlings in Colsess I, the factor pair $X_b x_b$ for yellow versus green seedlings in Black Hull-less, and the factor pair $A_h a_h$ for green versus white seedlings in Hanna, when in the heterozygous condition, had no effect on the following mature-plant characters: Number of culms, average length of culm, average length of head, number of grains per plant, and total grain yield per plant. With the factor pair $A_l a_l$ for white versus green seedlings in Canada Thorpe was associated an endosperm effect. In mature seeds borne on plants carrying the double-recessive factor pair $a_l a_l$, this endosperm effect was evident and reduced the yield. However the other plant characters were not affected.

MATERIAL AND METHODS

Colsess I[3] produced a white seedling in a monohybrid ratio. This seedling developed for about 10 days and then died.

Colsess II[3] produced a white seedling in a monohybrid ratio. This seedling behaved similarly to Colsess I, but differed from Colsess I genetically.

Colsess IV[3] produced a yellow seedling in a monohybrid ratio. This seedling grew under field conditions (low temperature) for about 10 days and then died. It has previously been reported as being linked with the factor pair $A_c a_c$ found in Colsess I. Crosses were made between Colsess I and Colsess IV in order to study the effect of the lethals in the heterozygous condition on some mature-plant characters. These characters were number of culms per plant, average length of culm per plant, average length of head per plant, number of grains per plant, and total weight of grain per plant. The segregation of the F_2 and F_3 plants again showed linkage.

[1] Received for publication Apr. 18, 1935; issued December 1935.

[2] ROBERTSON, D. W. THE EFFECT OF A LETHAL IN THE HETEROZYGOUS CONDITION ON BARLEY DEVELOPMENT. Colo. Agr. Expt. Sta. Tech. Bull. 1, 12 pp., illus. December 1932.

[3] ROBERTSON, D. W. LINKAGE STUDIES IN BARLEY. Genetics 14:1–36. 1929.

Crosses were also made between Colsess II and Colsess IV. The numbers found in the F_2 seedlings are shown in table 1.

The data indicate independent inheritance of the factor pairs $A_{c_3}a_{c_3}$ and X_cx_c. To further test the interrelationship of the factor pairs, the green F_2 plants were grown in F_3 and seedling counts made. The genetic constitution of the green F_2 plants as determined by the F_3 seedling behavior is given in table 2.

TABLE 1.—*Segregation of seedling color in the F_2 of a cross between Colsess II ($A_{c_3}a_{c_3}$) and Colsess IV (X_cx_c)*

Item	Green	Yellow	White
Observed number	1,025	335	431
Calculated segregation 9 : 3 : 4	1,007.44	335.81	447.75
	X^2=0.9347 P, very large		

TABLE 2.—*Genetic constitution of the F_2 green plants as determined by F_3 seedling numbers*

Item	Indicated genotypes			
	$A_{c_3}A_{c_3}X_cX_c$	$A_{c_3}a_{c_3}X_cX_c$	$A_{c_3}A_{c_3}X_cx_c$	$A_{c_3}a_{c_3}X_cx_c$
	Number	*Number*	*Number*	*Number*
Observed number	63	124	140	256
Calculated segregation 1 : 2 : 2 : 4	64.78	129.55	129.55	259.12
		χ^2=1.1672 P=0.7630		

The F_3 data give a good fit to a calculated 1 : 2 : 2 : 4 ratio for independent inheritance of the factor pairs $A_{c_3}a_{c_3}$ and X_cx_c.

The F_1 seeds were sown in 18-foot rows 1 foot apart, and spaced about 3 inches apart in the rows. Because of the presence of lethal seedlings in the F_2 population, the plants were harvested in two lots, the competitive plants, those growing adjacent to other plants in the rows being harvested separately. The remaining plants, the noncompetitive plants, with blank spaces adjacent to them, were also harvested separately. The presence of blanks in adjacent rows was not considered. After the growth data were obtained, 50 seeds from each F_2 plant were sown in moist sand, and the F_2 genetic constitution of the plants determined from F_3 seedling numbers.

EXPERIMENTAL RESULTS

A comparison was made of both the pure green ($A_{c_3}A_{c_3}$) and the heterozygous green and white ($A_{c_3}a_{c_3}$) F_2 plants in both the competitive and the noncompetitive groups. The plants were harvested and the measurements made during the winter of 1934–35. Fifty seeds from each plant were grown in the greenhouse to determine the genetic constitution of the plants for the seedling factors. The plants were then grouped according to their genetic constitution, and the data

analyzed. The results obtained from the green plants of the genetic constitution $A_{c_2}A_{c_3}$ and from the green plants heterozygous for the genetic factor pair $A_{c_3}a_{c_3}$ are given in table 3.

In the competitive plants no significant differences were found between the green and heterozygous plants. In the noncompetitive plants the same trend was found. The only significant difference was in the average length of culm, which, however, was in favor of the heterozygous plants. Since the trend was the same in both competitive and noncompetitive plants, only competitive plants were used in the study of the other factor combinations.

TABLE 3.—*Measurements of plant characters in Colsess II plants grown at Fort Collins, Colo.*

Characters studied	Segregation	Plants	Mean	Standard error of mean	Difference	Standard error of a difference	D/S. E. of difference	S. E. in percentage of mean
Competitive plants:		*Number*						*Percent*
Culms per plant, number.	Green	150	5.1533	0.1391	+0.1979	0.1956	1.0118	2.6992
	Heterozygous	168	5.3512	.1375				2.5695
Average length of culm, centimeters.	Green	150	62.5933	.4709	+.4543	.6559	.6296	.7523
	Heterozygous	168	63.0476	.4566				.7242
Average length of head, centimeters.	Green	150	6.8533	.0532	−.1003	.0745	1.3463	.7763
	Heterozygous	168	6.7530	.0521				.7715
Total grains, number	Green	150	210.3300	5.8412	−4.97	8.0920	.6142	2.7772
	Heterozygous	168	205.3600	5.6002				2.7270
Total weight of grain, grams.	Green	150	6.3260	.1889	−.1682	.2484	.6771	2.9889
	Heterozygous	168	6.1518	.1613				2.6220
Noncompetitive plants:								
Culms per plant, number.	Green	115	5.8609	.1905	+.1455	.2579	.5642	3.2504
	Heterozygous	157	6.0064	.1738				2.8936
Average length of culm, centimeters.	Green	115	61.5000	.5543	+1.5032	.7446	2.0188	.9013
	Heterozygous	157	63.0032	.4972				.7892
Average length of head, centimeters.	Green	115	7.0239	.0660	−.0287	.0867	.3310	.9396
	Heterozygous	157	6.9952	.0562				.8034
Total grains, number	Green	115	240.2200	7.9739	+7.07	10.8828	.6497	3.3194
	Heterozygous	157	247.2900	7.4063				2.9950
Total weight of grain, grams.	Green	115	7.6630	.2507	+.2813	.3474	.8097	3.2716
	Heterozygous	157	7.9443	.2405				3.0273

GREEN VERSUS YELLOW SEEDLINGS (X_cx_c)

Crosses involving Colsess IV (X_cx_c) were used in the study of green versus yellow seedlings. Table 4 gives the summarized data for the characters studied. The average length of head is the only one of the characters studied in the plants heterozygous for green versus yellow seedling color significantly different from the same characters in the homozygous green plants.

GREEN VERSUS WHITE SEEDLINGS (A_ca_c)

Heterozygous green plants (A_ca_c) and homozygous green plants (A_cA_c) were compared in crosses involving Colsess I. The data obtained from this study are also tabulated in table 4. None of the characters examined in the heterozygous plants differs statistically significantly from the same characters in the homozygous green plants. This indicates that the seedling factor pair $A_ca_{c_1}$, which produced green and white seedlings in Colsess I, is not detrimental to the growth of green plants having the genetic sonstitution A_ca_c.

TABLE 4.—*Measurements of plant characters in crosses involving Colsess IV and Colsess I (competitive plants), grown at Fort Collins, Colo.*

Characters studied	Segregation	Plants	Mean	Standard error of mean	Difference	Standard error of a difference	D/S. E. of difference	S. E. in percentage of mean
Colsess IV:		*Number*						*Percent*
Culms per plant, number.	Green	104	5.7308	0.1856	−0.1600	0.2306	0.6938	3.2386
	Heterozygous	219	5.5708	.1368				2.4557
Average length of culm, centimeters.	Green	104	62.3846	.6201	+.8323	.7261	1.1463	.9940
	Heterozygous	219	63.2169	.3777				.5975
Average length of head, centimeters.	Green	104	6.2885	.0669	+.4592	.0841	5.4602	1.0638
	Heterozygous	219	6.7477	.0510				.7558
Total grains, number	Green	104	207.2100	7.3518	+10.2558	9.0260	1.1363	3.5480
	Heterozygous	219	217.4700	5.2364				2.4079
Total weight of grain, grams.	Green	104	6.7500	.2280	−.0068	.2841	.0239	3.3778
	Heterozygous	219	6.7432	.1696				2.5151
Colsess I:								
Culms per plant, number.	Green	104	5.7308	.1856	+0.2317	.2269	1.0212	3.2386
	Heterozygous	160	5.9625	.1305				2.1887
Average length of culm, centimeters.	Green	104	62.3846	.6201	+1.6004	1.7937	2.1208	.9940
	Heterozygous	160	64.0750	.4954				.7732
Average length of head, centimeters.	Green	104	6.2885	.0669	+.1490	.0872	.7087	1.0638
	Heterozygous	160	6.4375	.0560				.8699
Total grains, number	Green	104	207.2100	7.3518	+9.04	9.1995	.9827	3.5480
	Heterozygous	160	216.2500	5.5300				2.5572
Total weight of grain, grams.	Green	104	6.7500	.2280	+.1438	.2814	.5100	3.3778
	Heterozygous	160	6.8938	.1649				2.3920

THE EFFECT OF TWO LETHAL FACTORS

The effect of two lethal factor pairs when in the heterozygous condition on the growth of plants was determined in families from a cross between Colsess II and Colsess IV. The factor pairs involved in this cross are $A_{c_3}a_{c_3}$ and X_cx_c. The data obtained from plants heterozygous for both factor pairs are given in table 5.

TABLE 5.—*Measurements of plant characters in competitive plants heterozygous for two lethal-factor pairs $A_{c_3}a_{c_3}$ and X_cx_c or for the two linked-factor pairs A_ca_c and X_cx_c*

Characters studied	Segregation	Plants	Mean	Standard error of mean	Difference	Standard error of a difference	D/S. E. of difference	S. E. in percentage of mean
$A_{c_3}a_{c_3}$ and X_cx_c:		*Number*						*Percent*
Culms per plant, number.	Green	150	5.1533	0.1391	+0.3632	0.2394	1.5171	2.6992
	Heterozygous	91	5.5165	.1949				3.5330
Average length of culm, centimeter.	Green	150	62.5933	.4709	−2.2362	.7451	3.0012	.7523
	Heterozygous	91	60.3571	.5774				.9566
Average length of head, centimeters.	Green	150	6.8533	.0532	−.1088	.0981	1.1091	.7763
	Heterozygous	91	6.7445	.0824				1.2217
Total grains, number	Green	150	210.3300	5.8412	−1.54	10.5705	.1457	2.7772
	Heterozygous	91	208.7900	8.8100				4.2196
Total weight of grain, grams.	Green	150	6.3200	.1889	+.3146	.3246	.9692	2.9889
	Heterozygous	91	6.6346	.2640				3.9791
A_ca_c and X_cx_c:								
Culms per plant, number.	Green	104	5.7308	.1856	+.4134	.2764	1.4957	3.2386
	Heterozygous	104	6.1442	.2048				3.3332
Average length of culm, centimeters.	Green	104	62.3846	.6201	+.9231	.8978	1.0282	0.9940
	Heterozygous	104	63.3077	.6493				1.0256
Average length of head, centimeters.	Green	104	6.2885	.0669	+.4327	.0989	4.3751	1.0638
	Heterozygous	104	6.7212	.0728				1.0831
Total grains, number	Green	104	207.2100	7.3518	+31.73	11.3440	2.7971	3.5480
	Heterozygous	104	238.9400	8.6390				3.6156
Weight of grain per plant, grams.	Green	104	6.7500	.2280	+1.0000	.3483	2.8711	3.3778
	Heterozygous	104	7.7500	.2632				3.3961

With the exception of the average length of culm, none of the differences between the characters studied in homozygous green plants and plants heterozygous for both factor pairs is significant. The green plants seem to have a higher average length of culm than the heterozygous plants. However, the general indications are that two lethals in the heterozygous condition are not detrimental to the growth of plants as indicated by the measurements of the number of culms, average length of head, total number of grains, and total grain weight.

A study of the linked genes in Colsess IV and Colsess I was made in the plants heterozygous for both of the factor pairs X_cx_c and A_ca_c. The data obtained from this study are also given in table 5.

In the study of the above-mentioned characters, it was found that all of the plants surviving in F_2 were heterozygous for both factor pairs. As the linkage of the factor pairs A_ca_c and X_cx_c shows less than 4 percent crossing over,[4] one would not expect any green plants in the small number of competitive plants studied. This made it impossible to compare the heterozygous plants with homozygous green plants of the same family. However, green plants from the families segregating for the single factor pairs X_cx_c and A_ca_c were used. The data in table 5 show significant differences in favor of the heterozygous plants for the following characters: Average length of head, total number of grains per plant, and total weight of grain per plant. The variability of the green and heterozygous plants is uniform for the same plant characters. This is indicated in the column headed S. E. in percentage of mean. A similar procedure was used in studying the relationship in the nonlinked factor pairs X_cx_c and $A_{c_3}a_{c_3}$, except that some green plants from the families segregating for both factor pairs were used. The discrepancy can hardly be explained on the basis of blank spaces in the adjacent rows since the same ratio of 9 green to 7 lethal seedlings was obtained in both families. The results indicate, as in the case of the nonlinked genes, that there is no detrimental effect of two lethal genes in the heterozygous condition on the growth characters studied in Colsess.

DISCUSSION

The data presented show less difference between the competitive and noncompetitive plants than was found in the previous study.[5] The same lack of detrimental effect is evident in both cases for the factor pair A_ca_c found in Colsess I.

The apparent difference in favor of the plants heterozygous for both the factor pairs A_ca_c and X_cx_c cannot be explained on the basis of the presence of lessened competition due to blank spaces in the adjacent rows since this condition was also present in the plants used for the study of the effect of the factor pairs $A_{c_3}a_{c_3}$ and X_cx_c, which are not linked. In the latter case no significant difference was found between the green plants of the genetic constitution $(A_{c_3}A_{c_3})$ (X_cX_c) and the above-mentioned green F_2 plants of the genetic constitution $(A_{c_3}a_{c_3})$ (X_cx_c). If the difference had been due to blank spaces in the adjacent rows the same difference should have been obtained in both families studied since the percentage of blank spaces was the same.

[4] ROBERTSON, D. W. See footnote 3.
[5] ROBERTSON, D. W. See footnote 2.

There is a possibility that the difference may be due to the recombining of dominant-growth genes linked with the lethal-factor pairs in Colsess I and Colsess IV.

SUMMARY

The effects of the lethal seedling-factor pairs $A_c a_c$, found in Colsess I, $A_{c_3} a_{c_3}$, found in Colsess II, and $X_c x_c$, found in Colsess IV, were studied as single heterozygotes in different plants and in combinations of double heterozygotes in different plants on the following mature-plant characters: Number of culms per plant, average length of culm per plant, average length of head per plant, total number of grains per plant, and total grain weight per plant.

Colsess I ($A_c a_c$) produced green and white seedlings in a 3 : 1 ratio. Colsess II ($A_{c_3} a_{c_3}$) also produced green and white seedlings in a 3 : 1 ratio, but is different genetical y from Colsess I. Colsess IV ($X_c x_c$) produced green and yellow seedlings in a 3 : 1 ratio and is linked to the factor pair ($A_c a_c$) in Colsess I.

No detrimental effects were found when plants of Colsess I, Colsess II, and Colsess IV in the heterozygous condition were compared with homozygous green plants of the same families.

No detrimental effects were found in the characters studied when plants containing the factor pairs ($X_c x_c$) ($A_{c_3} a_{c_3}$), double heterozygotes, were compared with pure green plants of the same families.

A similar lack of detrimental effect on the characters studied was found when plants containing the linked-factor pairs ($X_c x_c$) ($A_c a_c$), double heterozygotes, were compared with related green plants from the same cross.

SOIL-TEMPERATURE STUDIES ON FLORIDA CIGAR-WRAPPER TOBACCO [1]

By RANDALL R. KINCAID, *associate plant pathologist*, and L. O. GRATZ, *plant pathologist in charge, North Florida Substation of the Florida Agricultural Experiment Station*

INTRODUCTION

Numerous studies have been made to determine the effect of temperature on the growth of certain varieties of tobacco (*Nicotiana tabacum* L.), and on the development of certain diseases of this host. Several workers have studied the relation between soil temperature and germination of tobacco seed. Kincaid (*8*)[2] found that the cardinal temperatures for the germination of Florida cigar-wrapper tobacco seeds are approximately 10°, 24°, and 34° C.

The extent to which the growth of tobacco plants is affected by soil temperature apparently has received little attention. Johnson and Hartman (*5*) reported that white burley plants grew very little at temperatures below 13° C., best at 29° or 31°, and poorly at 40°; they noted that at the optimum temperature the plants grew low and stocky with broad but rather pointed leaves, while at temperatures near the maximum the plants were tall and spindly with short and rounded leaves. Godfrey (*2*), who also experimented with white burley tobacco, reported that there was a uniform increase in growth at temperatures from 10° to 25°, and some growth at 38°, the highest temperature which he used.

The effect of soil temperature on the development of certain soil-borne diseases of tobacco, especially black root rot, has received considerable attention, as is shown in a review by Jones, Johnson, and Dickson (*7*). These writers have also presented a valuable discussion of the relation of soil temperature to plant disease, making further discussion of this subject unnecessary here.

Tisdale and Kelley (*10*) made observations on the relationship between soil temperature and the development of black shank caused by *Phytophthora parasitica* var. *nicotianae* Tucker. They reported that plants transplanted early remained free of the disease for a few weeks, until the mean daily temperature of the soil reached about 20° C., and that the temperature during the remainder of the season never rose too high for infection

The cardinal temperatures for the growth on culture media of a strain of the black shank *Phytophthora* isolated at the North Florida Experiment Station are approximately 8°, 30°, and 36° C., according to data reported by Tisdale and Kelley (*10*) and Tucker (*11*).

Black shank is the most important disease of tobacco in the district of northern Florida and southwestern Georgia where cigar-wrapper

[1] Received for publication May 13, 1935; issued December 1935.
[2] Reference is made by number (italic) to Literature Cited, p. 449.

tobacco is grown under shade. This disease was first identified in the United States by Tisdale (*9*) in 1922, and by breeding and selection he produced varieties of cigar-wrapper tobacco highly resistant to it.

The object of the investigation reported below was to determine the cardinal soil temperatures both for the development of the black shank disease and for the growth of Florida cigar-wrapper tobacco plants. Some observations were made on the relation of soil temperature to the development of black shank in the field.

APPARATUS, METHODS, AND MATERIALS

The apparatus used in these studies for the control of soil temperature was similar to that described by Camp and Walker (*1*). It consisted of eight compartments filled with water. Each compartment accommodated eight cans of soil, and the level of the water outside the cans was about the same as the level of the soil inside the

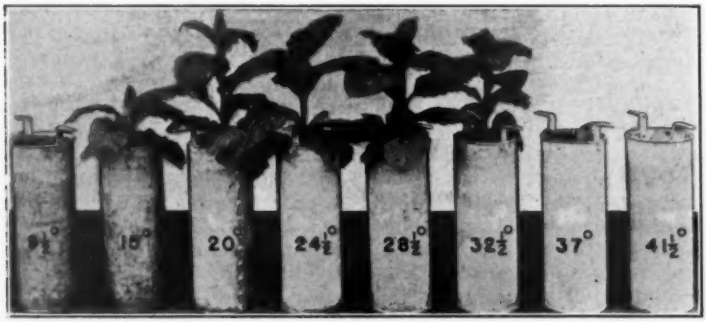

FIGURE 1.—Effect of constant soil temperatures (indicated on the cans) on the growth of tobacco plants of the Round Tip variety; see table 1, trial 2.

cans. An agitator in each compartment kept the water in continuous circulation. The compartment at one end of the apparatus was cooled by means of a thermostatically controlled refrigerator, and the compartment at the other end was heated by means of thermostatically controlled electric heaters of the immersion type. The other six compartments adjusted themselves to a series of intermediate temperatures, differing by 3° to 5°.

The cans (fig. 1), made of galvanized iron, were 8 inches in diameter and 22 inches deep, and had a capacity of about 4 gallons of soil. They were brought to equal tare by the addition of coarse gravel, and the same weight of moist soil was added to each.

The soil was a fine sandy loam having a pH value of about 5.2. The fertilizer used was composed of 100 pounds of cottonseed meal, 10 pounds of precipitated bone, and 15 pounds of sulphate of potash, and analyzed 8 percent NH_3, 8 percent P_2O_5, and 5 percent K_2O. It was applied at the rate of 5 ounces per 4-gallon can. Before the fertilized soil was used in these experiments, it was heated in an oven for about 6 hours at 60° to 70° C., or for about 2 hours at 80° to 90°, to destroy the black shank fungus and root knot nematodes.

The water-holding capacity of the soil was determined by the Hilgard (*4*) method, and the moisture content by drying samples in an oven at 105°. The initial water content of the soil in the different trials varied from 14 to 19 percent of the dry weight, or from 32 to 40 percent of the water-holding capacity. In the first two trials, the water content of the soil in the coldest compartment increased to about 25 percent of the dry weight as a result of the condensation of atmospheric moisture on the cold inner surface of the cans. However, the appearance of the plants in the cans was normal, and the difference in the moisture content of the soil was not believed to be of much importance. A 1-inch layer of coarsely granulated cork was added to the top of the soil after the plants had been transplanted, for the purpose of insulation.

The temperatures observed in the soil at a depth of 4 inches seldom varied more than 1° above or below the average temperatures reported in the tables. The mean daily temperature of the greenhouse in which the apparatus was located ranged from 23° C. in one trial conducted in midwinter to 29° C. in another trial conducted in early summer, and the daily range was generally between 10° and 15°. Conditions in the greenhouse during all trials were favorable for the growth of tobacco plants. The cans were weighed once a week, and sufficient tap water was added to the soil once or twice a week to restore them to their original weight, but no allowance was made for the weight of the plants.

The plants used in these experiments were of the variety No. 301, which is resistant, and Round Tip,[3] which is highly susceptible to black shank. No. 301 was developed at this station by Tisdale and Round Tip was developed in Connecticut by Hayes, East, and Beinhart and by Jones (*3*, *6*). Seed plants had been self-pollinated for several successive generations, and the seeds used were probably pure lines. The seedlings were grown in flats of sterilized soil. They were carefully selected for uniformity of size and color, and one seedling was transplanted to each can. The height of the plants at the time of transplanting was about 1½ inches.

THE CARDINAL SOIL TEMPERATURES FOR THE GROWTH OF TRANSPLANTED TOBACCO SEEDLINGS

Several trials were conducted to determine the cardinal soil temperatures for the growth of cigar-wrapper tobacco plants for a period of a few weeks after transplanting. These temperatures may be defined as follows: Minimum, the lowest temperature at which growth occurs; optimum, the temperature at which the most growth occurs; maximum, the highest temperature at which growth occurs.

A few days after the seedlings had been transplanted, the cans were placed in the tanks and brought to the various constant temperatures. After a certain period of growth, observations were made on the plants as follows: (1) Height from the soil to the terminal bud, (2) length of the longest leaf, (3) number of leaves longer than 1

[3] Both of these varieties may be classified under type 62,[4] which comprises southern shade tobacco grown for cigar wrappers.

[4] UNITED STATES DEPARTMENT OF AGRICULTURE, BUREAU OF AGRICULTURAL ECONOMICS. STANDARD GRADES FOR SOUTHERN SHADE TOBACCO (U. S. TYPE 62). 15 pp. 1933. [Mimeographed.]

inch, and (4) the green weight of the plants. Measurements of green weight were made only on the plants of No. 301; the Round Tip plants were used for the studies on black shank.

A preliminary trial was conducted with Round Tip plants to obtain an indication of the limits of temperature for growth. Because of

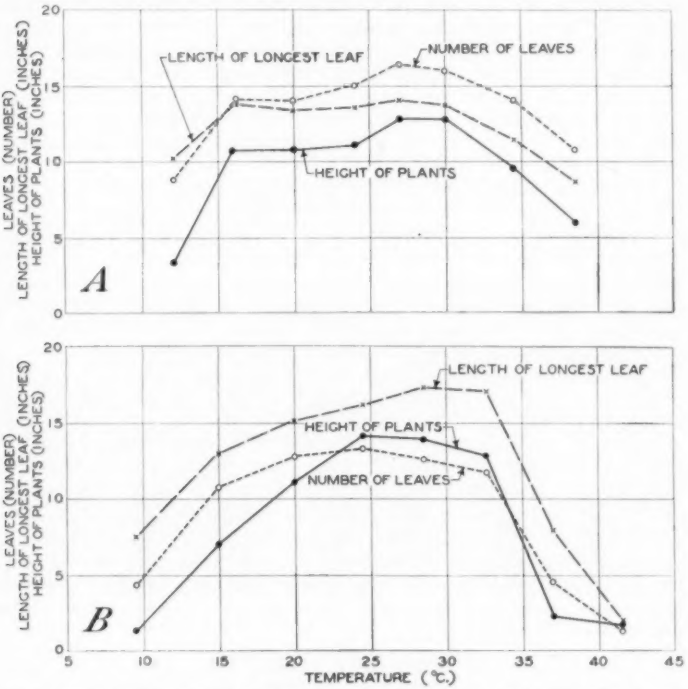

FIGURE 2.—Effect of various constant soil temperatures on the growth of tobacco plants of the Round Tip variety: *A*, Trial 1; *B*, trial 2.

mechanical difficulties incidental to the adjustment of the refrigerator, the control at the cold end of the apparatus was poor. However, it was evident from the results that the minimum was slightly below the lowest temperature used, 16° C. and below, and that the maximum was approximately 40°.

Three other trials with Round Tip and two trials with no. 301 plants were conducted. The average measurements of the plants in these five trials are given in table 1. The growth of Round Tip plants in trial 2 is shown in figure 1; the plants selected for the photograph were those which best illustrated the average measurements of

the plants grown in the respective compartments. The growth of the plants in trials 1, 2, 4, and 5 is shown graphically in figures 2 and 3.

These results indicate that the minimum temperature for growth of transplanted seedlings of both Round Tip and No. 301 is about 9° C.,

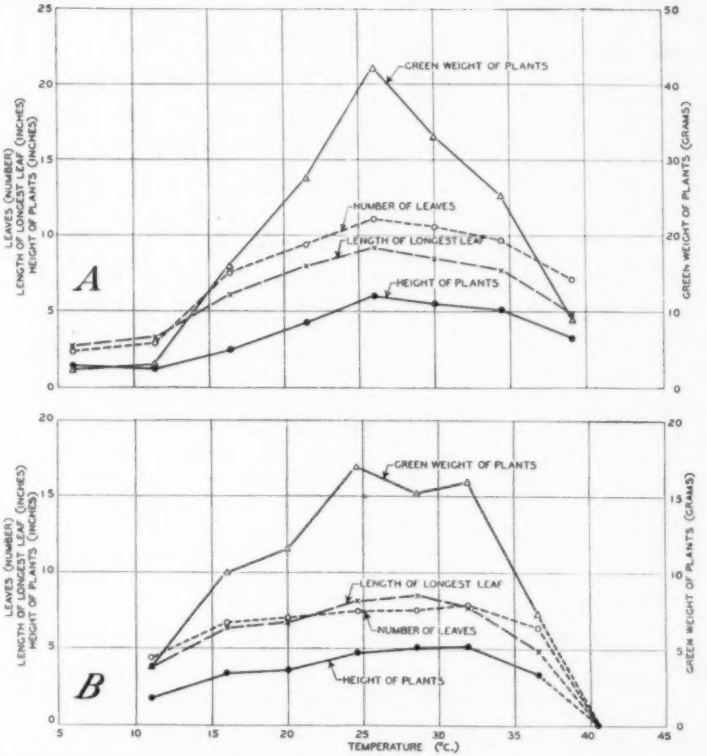

FIGURE 3.—Effect of various constant soil temperatures on the growth of tobacco plants of variety No. 301: *A*, Trial 4; *B*, trial 5.

and the maximum about 40°. The optimum was not very closely defined, because the best growth, as indicated by the measurements, was distributed over a range of temperatures from 24½° to 32°.

The minimum for the growth of transplanted seedlings corresponds closely to the minimum for the germination of seeds of No. 301 (*8*) but the optimum and maximum are somewhat higher.

TABLE 1.—*Effect of various constant soil temperatures on the growth of tobacco plants of the Round Tip and No. 301 varieties*

Trial no.	Variety	Period of observation	Soil temperature	Plants	Average height of plants	Average length of longest leaf	Average number of leaves	Average green weight per plant
		Days	*° C.*	*Number*	*Inches*	*Inches*		*Grams*
1	Round Tip	47	12	7	3.3	10.1	8.7	
			16	[1] 6	10.7	13.9	14.0	
			20	[1] 6	10.7	13.3	14.0	
			24	7	11.0	13.5	15.0	
			27	7	12.8	14.0	16.4	
			30	7	12.8	13.7	16.0	
			34½	7	9.6	11.3	14.0	
			38½	7	6.0	8.6	10.7	
2	do	32	9½	7	1.3	7.3	4.4	
			15	7	7.1	13.0	10.8	
			20	7	11.1	15.1	12.8	
			24½	7	14.0	16.1	13.3	
			28½	7	13.9	17.3	12.7	
			32½	7	12.8	17.1	11.8	
			37	7	2.3	7.9	4.6	
			[2] 41½	7	1.8	1.9	1.4	
3	do	49	10½	1	9.5	8.5	11.0	
			16	1	17.0	12.5	17.0	
			20	1	18.5	13.5	18.0	
			24½	1	18.5	14.5	17.0	
			28	1	21.5	14.0	20.0	
			31½	1	21.5	13.0	18.0	
			35	1	13.5	10.5	13.0	
			38½	1	4.0	4.5	5.0	
4	No. 301	31	6	7	1.5	2.8	2.4	2.4
			11½	7	1.3	3.3	3.0	2.9
			16½	7	2.7	6.1	7.7	16.1
			21½	7	4.3	8.1	9.7	27.6
			26	7	6.1	9.5	11.1	42.1
			30	7	5.6	8.7	10.7	33.3
			34½	7	5.1	7.9	9.9	25.7
			39	7	3.4	4.9	7.1	9.3
5	do	21	14	8	1.8	3.9	4.4	3.9
			16	7	3.3	6.4	6.6	10.0
			20	7	3.6	6.8	7.0	11.7
			24½	7	4.8	8.0	7.6	17.0
			28½	7	5.1	8.3	7.6	15.3
			32	7	5.2	7.7	7.9	16.0
			36½	7	3.4	4.9	6.4	7.3
			[3] 40½	8				

[1] 1 plant accidentally killed. [2] Plants nearly dead. [3] All plants dead or dying.

EFFECT OF CONSTANT SOIL TEMPERATURE ON THE DEVELOPMENT OF BLACK SHANK

Four trials were conducted to determine the cardinal soil temperatures for the development of black shank caused by *Phytophthora parasitica* var. *nicotianae* Tucker (*11*). These temperatures may be defined as follows: Minimum, the lowest temperature at which symptoms of the disease appear; optimum, the temperature at which symptoms of the disease appear in the largest percentage of the plants within the shortest time after inoculation; maximum, the highest temperature at which symptoms of the disease appear.

Seven transplanted seedlings of the Round Tip variety, which is very susceptible to black shank, were inoculated and incubated at each temperature for each trial. The fungus used originated from a single-spore culture of *Phytopthora* isolated from tobacco at this station, and was grown on potato-dextrose agar or on steamed wheat. The fungus and the medium were mixed with the soil shortly before transplanting in two trials, and placed in contact with the stems below the surface of the soil in two others. The amount of inoculum was the same in all cans of each experiment, and was evidently sufficient in every trial to insure prompt infection at favorable tem-

peratures. One noninoculated check plant per compartment was left in each trial.

The plants used in the first three trials were placed in the cans in blocks of soil in which they had been transplanted a few days previously, so that the growth of the plants was not seriously interrupted. The plants used in the last trial were pulled and transplanted according to the usual field practice.

The time which elapsed between inoculation and the appearance of the symptoms of the disease was carefully observed. Diseased plants were recognized by permanent wilting, generally followed by the blackening of the stem above the soil and cork. Stunting of the inoculated plants, as compared with the check plant at the same temperature, was often noticeable before the wilting; the checks remained healthy in every instance.

The results of four trials with Round Tip plants are given in table 2. That temperature has an important effect on the pathogenic activity of the fungus is shown by the number of plants infected and the time required for the appearance of symptoms of the disease. The minimum temperature varied considerably with the age of the plants, from 16° C. or lower for newly transplanted seedlings to about 24° for plants inoculated 41 or 47 days after transplanting. The optimum was approximately 28°. The maximum was near 34°, but was not clearly defined, for a few plants died apparently from the combined effects of black shank and unfavorably high temperature.

TABLE 2.—*Effect of various constant soil temperatures on the development of black shank in tobacco plants of the Round Tip variety*

Trial No.	Soil temperature	Period of incubation before inoculation	Plants showing symptoms of black shank after indicated number of days						
			4	6	10	14	15	20	40
	°C.	*Days*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*
1	12	47		0				0	
	16			0				0	
	20			0				0	
	24			0				2	
	27			2				6	
	30			4				6	
	34½			4				5	
	38½			0				1	
2	9½	41		0			0		
	15			0			0		
	20			0			0		
	24½			0			1		
	28½			6			6		
	32½			4			6		
	37			0			0		
	41½			(1)			(1)		
3	10½	0			0				0
	16				0				0
	20				0				2
	24½				0				6
	28				5				6
	31½				2				6
	35				0				0
	38½				0				0
4	16	0	0	0	0	3	3		
	19		0	0	3	4	5		
	22½		0	0	2	5	6		
	25½		1	3	6	6	6		
	28		1	4	6	6	6		
	31½		0	0	3	3	4		
	34		1	1	1	1	1		
	37		0	0	1	2	2		

[1] Plants nearly dead at time of inoculation.

The effect of soil temperature on the development of the disease is probably due to the effect of temperature on the rate of growth of the fungus either saprophytically in the soil or parasitically in the tissues of the host. In fact, the cardinal temperatures for the development of the disease correspond somewhat closely to those reported by Tisdale and Kelley (*10*) and Tucker (*11*) for the growth on culture media of the pathogen isolated by Tisdale at this station.

The number of plants available for observation in the experiments reported was limited by the capacity of the apparatus, but the general agreement of the results of the various trials indicates that the conclusions are fairly reliable. The plants inoculated several weeks after transplanting (table 2, trials 1 and 2) were grown at the same temperature at which they were held after inoculation. It is possible that the temperature at which the plants were grown had some influence on their susceptibility. This subject warrants further investigation.

RELATION OF SOIL TEMPERATURE TO THE DEVELOPMENT OF BLACK SHANK IN THE FIELD

The results of the experiments reported above, as well as those of Tisdale and Kelley (*10*), show that the temperature of the soil may affect the activity of the black shank organism in the field. Some further field observations may be of interest here.

In September 1932, Round Tip plants were transplanted in the trial plot at this station, where high percentage of the Round Tip plants in the check rows had died during the usual growing season which ended in July. These plants were still free from black shank when they were pulled for examination in November. This indicates that the pathogen had become inactive in the soil during the midsummer months. No soil-temperature records for these months are available. The following season, Round Tip plants transplanted in the same field in March remained healthy for several weeks. The first wilted plants were observed about the first of May, when the soil temperature at a depth of 2 to 4 inches ranged from about 20° C. at night to 24° during the day. This agrees closely with the minimum temperature (24°) reported above for the development of black shank in Round Tip plants inoculated several weeks after transplanting.

The problem of the survival, multiplication, and distribution of the black shank fungus under field conditions deserves further investigation.

SUMMARY AND CONCLUSIONS

Experiments were conducted to determine the cardinal soil temperatures for the growth of transplanted cigar-wrapper tobacco seedlings. The minimum and maximum were found to be approximately 9° and 40° C., respectively, and the optimal range from about 24½° to 32°.

Experiments were conducted to determine the effect of constant soil temperatures on the development of black shank (*Phytophthora parasitica* var. *nicotianae* Tucker) in Round Tip tobacco plants, which are very susceptible to the disease. The minimum temperature for infection was found to vary considerably with the age of the plants, ranging from 16° C. or lower for newly transplanted seedlings to about 24° for plants inoculated several weeks after transplanting. The optimum is about 28° and the maximum about 34°.

Observations on black shank in the field indicate that soil temperature is an important factor in the development of the disease.

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THE DEVELOPMENT OF THE BARLEY SPIKE [1]

By O. T. BONNETT

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INTRODUCTION

A study of the morphological development of the barley spike has practical significance for the agronomist and should be of interest to the botanist. From a study of the morphological development of the barley spike the agronomist can determine the critical period at which spike development may be affected by the environment. Differences in the rate of differentiation and growth in early spike development give a suggestion of the reasons for variation in the mature spike. Finally, the manner of the adjustment of the spike to environmental changes is indicated. To the botanist the development of the barley spike furnishes an example of the sequence of differentiation in a grass stem.

Publications dealing with the development of the barley stem from germination to pollination are few. Two publications that deal with the development of the barley spike from the earliest stages to complete differentiation should be mentioned. Schuster [2] published a description and a set of drawings illustrating several steps in spike and spikelet development. More recently Noguchi [3] has given a brief description of the spike and spikelet development and has illustrated some of the phases of development by drawings. He also gives data showing the length and breadth of the spike of 6-row and 2-row barley at different stages from 10 to 200 days old.

The illustrations accompanying the above-mentioned publications are line drawings and hence present more or less diagrammatically what is shown in this article by photographs. In addition, several stages of development are shown in this article that are not included by the above-mentioned authors.

MATERIALS AND METHODS

Both 2-row and 6-row barley were used in this study and the photomicrographs that best showed the successive steps in spike and spikelet development were chosen without regard to type. This was justified since both types follow the same sequence of development, the only exception being that the side spikelets of the 2-row barley do not develop as rapidly or as completely as the central spikelets.

The plants were grown in pots in the greenhouse, and when they were 20 days old daylight was supplemented with electric light from 500-watt bulbs placed about 3½ feet above the bench on alternate nights. These conditions of growth produced normal plants except that the electric light hastened the development of the spike.

[1] Received for publication Apr. 15, 1935; issued December 1935.

[2] SCHUSTER, J. ÜBER DIE MORPHOLOGIE DER GRASBLUTE. Flora [Jena] 100: 213–266, illus. 1910.

[3] NOGUCHI, Y. STUDIEN ÜBER DIE ENTWICKLUNG DER INFLORESZENZEN UND DER BLUTEN BEI GETREIDEPFLANZEN. Jour. Col. Agr., Imp. Univ. Tokyo 10: 247–303, illus. 1929.

At intervals of a few days, plants were pulled, taken to the laboratory, and the growing points dissected from the stem under the low power of a binocular microscope. Especially ground dissecting needles were used. The growing point was easily exposed and removed for photographing by carefully cutting and removing the leaves enclosing it.

Photomicrographs were taken with an upright camera, adjusted to fit over one side of the binocular microscope. It was necessary to construct a special light-tight collar to connect the microscope and camera. The eyepiece was left in the microscope when exposures were made.

Light for photographing was obtained from a microscope lamp fitted with a 200-watt bulb. A Florence flask filled with water containing sufficient copper sulphate to produce a light blue-green color was used as a condenser. This set-up gave a light of sufficient intensity to produce a good negative on commercial orthochromatic film with about 10 seconds' exposure.

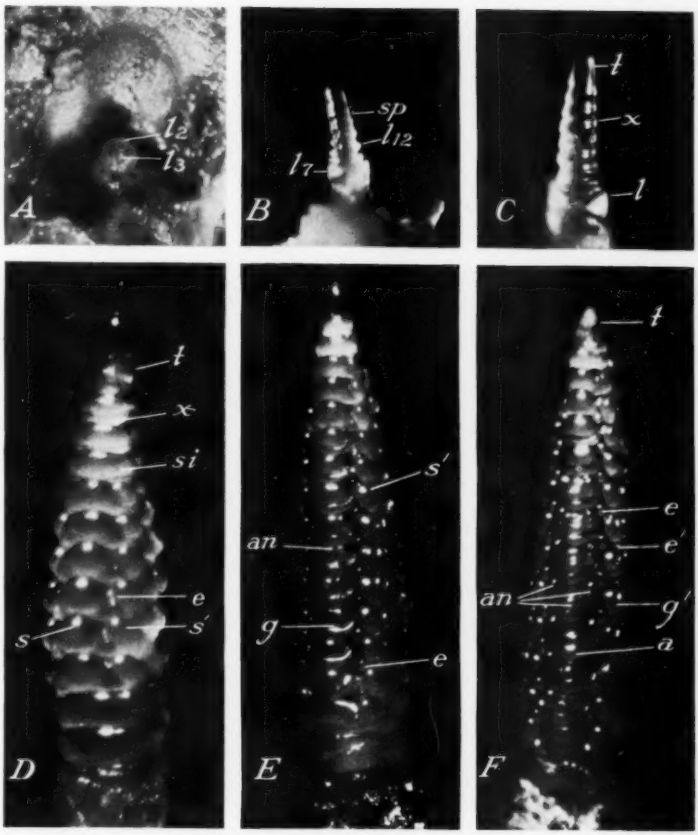
At first great difficulty was experienced in making photographs because of the very rapid drying of the growing points. Later, the growing points when dissected were placed on moist blotting paper in a small preparation dish and stored in a Petri dish lined with wet paper towels. Material handled in this way could be kept for 24 hours without any apparent deterioration. It was also found that specimens stored in the moist chamber for a period of time did not dry out as rapidly as those that had just been dissected.

Two methods of mounting the growing points for photographing were used. In one the specimen was mounted on moist blotting paper over black paper placed in the bottom of a preparation dish. The preparation dish was covered with a watch glass for the preliminary manipulations and focusing and then the watch glass was removed for the final focusing. The other method was to place a drop of petroleum jelly on a glass slide, mount the specimen in the jelly, and then place the slide over a black velvet background. Both methods of mounting were satisfactory except that in using the latter method more speed was necessary to complete the photographing before the specimen dried.

Growing points of barley are very difficult to photograph because they are colorless and nearly transparent. They were photographed against a black background with the light so placed that the high lights and shadows brought out the detail. In some cases in order to provide contrast, a stain composed of a mixture of 90-percent alcohol, a small amount of glycerin and basic fuchsin was applied to the specimen with a camel's-hair brush. The alcohol quickly evaporated, leaving the glycerin and stain in the folds of the various structures. Since the red stain photographed black the details of the structures were clearly outlined.

DESCRIPTION OF SPIKE DEVELOPMENT

In the resting stage of the barley grain the stem of the embryo is composed of only a few structures. These are the coleoptile and first leaf, which are the largest of the structures, the second and third leaf initials, the growing point, and a tiller bud in the axil of the coleoptile. The coleoptile and first leaf have been dissected from the embryo of the barley seed in plate 1, *A*, to show the second and third leaf



A, A part of the embryo of a kernel of barley with the coleoptile and first leaf removed: l_2, Second leaf; l_3, third leaf. × 21.

B, Growing point of a barley stem in the 3-leaf stage: l_7, Seventh-leaf initial; l_{12}, twelfth-leaf initial; *sp.*, spike primordium. × 17.

C, Growing point of a 6-row barley stem in the 4-leaf stage, showing double ridges marking the beginning of spike differentiation: *l*, Leaf initial; *x*, double ridge; *t*, tip of spike. × 25.

D, Young spike of 6-row barley from a stem in the 5-leaf stage, showing the beginning of spikelet formation: *s*, Central spikelet; *s′*, side spikelet; *e*, empty glume; *si*, spikelet initial; *x*, lower of a double ridge; *t*, tip of spike. × 40.

E, Spike of a 6-row barley stem in the 5-leaf stage when the lemma and anthers begin to form. Stain has been used to make clear the position of the spike structures: *e*, Empty glume; *g*, lemma; *an*, anthers; *s′*, side spikelet. × 25.

F, A spike of 2-row barley from a stem in the 6-leaf stage showing a more advanced stage of glume, anther, and awn development. Stain has been used to mark out the structures: *a*, Awn; *an*, anthers; *g′*, lemma of a side spikelet; *e′*, and *e*, empty glume initials of the side and central spikelets respectively; *t*, tip of spike partially dried. × 25.

initials. The growing point, which is hemispherical, is partly enclosed by the third leaf initial. By carefully removing the second and third leaves the primordium of the fourth leaf can be seen as a transverse ridge of the growing point.

By the time the second leaf is well grown nearly all of the leaves and leaf initials that the main stem will have can be found. The leaves range in size from those fully grown to leaf primordia just distinguishable as ridges at the base of the growing point. At this stage of stem development the growing point has just begun to elongate in preparation for spike differentiation. Up to this time the growing point has been short and hemispherical.

The growing point of a stem in the three-leaf stage is shown in plate 1, *B*. The leaves that enclosed this growing point ranged in size from the fully grown first and second leaves with the third leaf about 2 inches long down to the sixth leaf that was just large enough to enclose the growing point. The seventh-leaf initial is the basal one (pl. 1, *B*, l_7), while the last prominent ridge (pl. 1, *B*, l_{12}) is probably the last leaf that the stem would have produced. Above the twelfth-leaf initial is the part of the growing point from which the spike is differentiated.

The first indication of spike differentiation is the appearance of double ridges (pl. 1, *C*, *x*) instead of single ridges as was noted previously. At first the ridges are nearly equal in size, but the upper ridge of each pair grows more rapidly and from it the spikelets are formed. The lower ridge of the pair probably becomes the internode of the rachis, for apparently all of the spikelet structures arise from the upper of the pair of ridges.

Spikelet differentiation is first indicated by the appearance of two slight depressions in the transverse meristematic ridge. The very earliest stages of spikelet differentiation can be noted in the two spikelet initials at the base of the spike (pl. 1, *D*). Growth occurs in both sides and between the two furrows in preparation for the differentiation of the spikelet parts. Soon two little papillae, or empty glume initials, appear on opposite sides of each spikelet (pl. 1, *D*, *e*), but they appear first on the central spikelets.

Several stages of spikelet development are shown on the same spike (pl. 1, *D*). In the center portion of the spike the spikelet initials are quite prominent. The two transverse ridges at the base of the spike and some of the ridges at the top of the spike show only the first evidences of spikelet differentiation. The very tip of the spike is smooth and shows little evidence of the formation of ridges, while in plate 1, *D*, *x*, the lower of a pair of ridges can be seen.

The lemma is the first structure of the spikelet to differentiate (pl. 1, *E*, *g*). It appears as a distinct ridge across the spikelet initial and forms first upon the central spikelets in the central portion of the spike. Differentiation of the palea occurs somewhat later than that of the lemma, but since it is hidden by the other spikelet parts its development cannot be followed in gross dissections.

Soon three little papillae appear upon the meristem above the lemma (pl. 1, *E*, *an*, and *F*, *an*). These little papillae are the primordia of the anthers. The pistil is formed from that portion of the meristem located between the anthers, but it does not differentiate till considerably later than the anthers. The ovary differentiates

first, followed by the styles and last, the stigma, but no features of the differentiation of the pistil can be seen in gross dissections.

A side view of a young spike of barley is presented in plate 2, *A*, for the purpose of showing an early stage in the development of the rachis. In the early stages the internodes of the rachis are very short, but as the spike matures the internodes elongate. The degree of elongation determines spike density, a character used in the classification of barley varieties.

The awn begins its development as an outgrowth from the lemma (pl. 1, *F*, *a*). The awns and anthers grow quite rapidly and together with the empty glumes are soon the most conspicuous of the spikelet structures (pl. 2, *B* and *C*). The awn grows much more rapidly than the lemma and palea, which remain short with the anthers extending above them. About the time the last internode of the stem begins to elongate the glumes begin their growth and finally enclose the anthers and pistil. It should also be mentioned that in barbed varieties of barley the barbs can be seen on the awn at an early stage.

While the spike of barley shown in plate 2, *B*, was chosen principally to show the early development of the awn, it also shows the differentiation of a 6-row barley at the 6-leaf stage. A difference in development at the base and tip of the spike should be noted as well as the relative stages of differentiation in the side and central spikelets.

The relative size and degree of development of the tip of the spike and the spikelets a little lower on the spike should be noted (pl. 2, *C*, *t*). The very tip of the spike is at this stage a bit of undifferentiated meristem, and the spikelets at the uppermost 7 or 8 nodes of the rachis, as contrasted with the spikelets below, are much retarded in development. As has been pointed out, in the early stages of development the tip of the spike remains undifferentiated and smooth in outline. As the spike approaches maturity spikelet differentiation proceeds apically, but the last-formed spikelets never complete development, always remaining infertile and rudimentary. Rudimentary spikelets at the tip of the spike can be seen on any mature barley spike.

At the base of the spike is a structure called the collar (pl. 2, *D*, *c*). This structure is a distinct ridge of tissue circling the stem at the first node of the spike. A similar but less prominent ridge of tissue is found at the first node above the collar (pl. 2, *D*, *x*). So far as has been determined in these studies, the collar and the ridge at the node above are formed by two leaf initials that are just beginning to differentiate but are not far enough along to continue their develop-

EXPLANATORY LEGEND FOR PLATE 2

A, Spike of a 2-row barley stem in the 6-leaf stage, showing the rachis and a side view of anthers, awns, and side spikelets. Stain has been used to mark out structures: *an*, Anthers; *a*, awn; *s'*, side spikelet. × 30.

B, A 6-row barley spike from a stem in the 6-leaf stage, illustrating awn development and the comparative development of side and central spikelets: *a*, Awn; *an*, anthers; *s*, central spikelet; *s'*, side spikelet; *t*, tip of spike. × 25.

C, Part of a barley spike from the stem of a 2-row barley in the 5-leaf stage, showing the differentiation of the tip of the spike: *an*, Anthers; *e*, empty glume; *e'*, empty glume of side spikelet; *s'*, side spikelet; *a*, awn; *t*, tip of spike. × 25.

D, Part of a spike from a stem in the 6-leaf stage, showing the collar at the base of the spike: *c*, Collar; *x*, second node of the rachis. × 30.

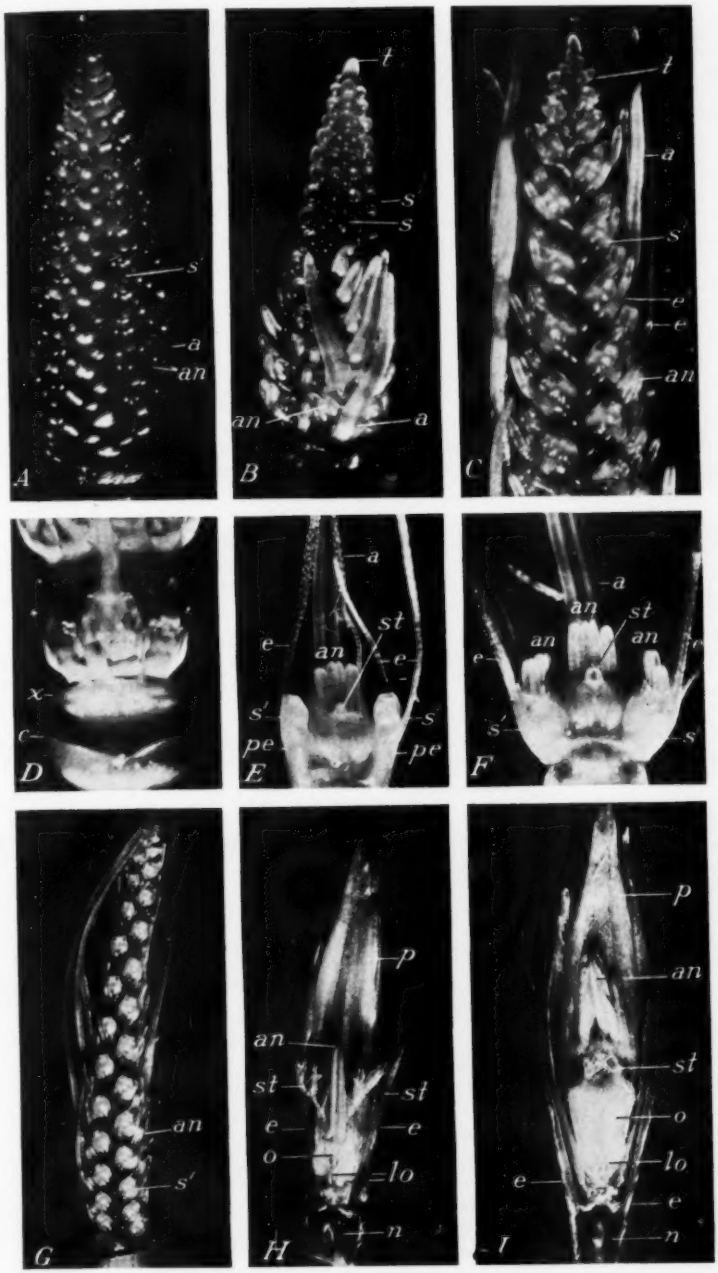
E, Spikelet of a 2-row barley: *pe*, Pedicel; *s'*, side spikelet; *st*, style; *e*, empty glumes; *an*, anthers; *a*, awn. × 15.

F, Spikelet of a 6-row barley: *s'*, Side spikelet; *e*, empty glume; *st*, style; *an*, anthers; *a*, awn. × 15.

G, Spike of a 2-row barley: *s'*, Side spikelet; *an*, anther. × 8.

H, Spikelet before pollination: *r*, Rachis; *lo*, lodicules; *o*, ovary; *e*, empty glumes; *st*, stigma; *an*, anther; *p*, palea. × 5.

I, Spikelet after pollination: legend as for *H*.



FOR EXPLANATORY LEGEND SEE OPPOSITE PAGE.

ment as leaves at the time spikelet differentiation begins. They are arrested in their development and form the structures mentioned. While spikelets develop at the collar, they are late and usually rudimentary and sterile.

A spike of a 2-row barley well advanced in its development is shown in plate 2, *G*. The awns on the central spikelets are well developed but not fully grown and the anthers protrude well beyond the glumes. The side spikelets are small, without awns on the lemma, and rudimentary anthers extend slightly beyond the glumes. Much in evidence are the empty glumes upon which the barbs can be seen. Barbs can also be seen on the awns.

A spikelet of a 2-row barley (pl. 2, *E*) and a spikelet of a 6-row barley (pl. 2, *F*) are shown for comparison. Both spikelets were taken from stems in about the same stage of development. The last internode of the stem, the one to which the spike is attached, had just begun to elongate. The anthers and stigmas in the spikelets of the 6-row and in the central spikelet of the 2-row barley extend above the flowering glumes. However, just before the head emerges from the boot, the lemma and palea which up to this time have grown slowly begin to grow rapidly and soon enclose the anthers and stigmas. The anthers in the side spikelets of the 2-row barley have been enclosed by the flowering glumes. The side spikelets of the 2-row barley are much smaller than the central spikelet, are without awns on the lemmas, and are pedicellate. The side spikelets of the 6-row barley are nearly as large as the central spikelet, have awns on the lemmas, and are sessile. The side spikelets of the 6-row barley are fertile, while those of the 2-row barley are sterile. The stigmas in neither have branched (pl. 2, *E*, *F*, *st*).

Mature flowers before pollination and after pollination are shown in plate 2, H, and plate 2, I, respectively. The lemmas have been cut away to show the flower parts. Only one anther can be seen (pl. 2, *H*). The other two anthers are hidden in the folds of the palea. Before pollination the anther is not dehisced, the stigmas are erect, branched, and feathery, the lodicules swollen and turgid, and the ovary small. After pollination the anthers are dehisced, the stigmas are collapsed, the lodicules are shrunken, and the ovary has increased in size. The fertilized ovary after a period of growth and differentiation becomes the barley kernel.

Aside from the pistil and palea mentioned previously, two other spikelet structures, the rachilla and the lodicules, have not been followed in their differentiation and development in this study. They are so located that their differentiation and development cannot be shown.

DISCUSSION

That period in the morphological development of a head-bearing barley stem extending from germination to pollination can be divided into two phases. These phases can be determined approximately by examining the stem and more accurately by examining the growing point. In the first phase of development, the internodes of the stem do not elongate, leaves grow, leaf initials are the only structures differentiated from the growing point, and the growing point above the base remains smooth in outline but increases in length. The changes in the stem and the growing point which mark the transition

from the first to the second stage are shown by the beginning of internode elongation in the stem and the appearance of double ridges on the growing point. In the second phase of development, the internodes of the stem elongate, the spikelets and spikelet structures differentiate, increase in size, and complete their development in preparation for pollination.

It is interesting to note how the phases of stem development of a barley plant parallel each other not only in those stems that produce heads but in all stems even down to the tiller buds. When stems producing heads pass into the second phase, i. e., jointing and spike differentiation, it is not long before all stems on the plant follow in rapid succession. An examination of a plant in head shows even the growing point of the tiller buds to be in the process of spikelet differentiation.

Very early differences in the time of differentiation and rate of spikelet development are maintained and are reflected in the mature spike. Referring to plate 1, *D*, *E*, and *F*, the early differences will be noted. The spikelets in the middle of the spike are in advance of the basal spikelets and the basal spikelets are in advance of the tip, which is the last portion of the spike to differentiate. The central spikelets are more advanced in development than the side spikelets in both the 2-row and 6-row types. All of these differences are reflected in the mature spike. The best developed and heaviest kernels are in the middle portion of the spike, the basal kernels next, and the tip kernels are the lightest of all. The kernels in the central spikelets of the 6-row barley are heavier than those in the side spikelets. While the spikelets progressively develop at the tip of the spike, the spikelets remain rudimentary and do not bear kernels. Thus those spikelets that have an initial advantage in differentiation maintain this advantage throughout spike development.

Since the number of spikelets at the joints of the rachis is fixed, response to the environment during early differentiation takes place principally at the tip of the spike. The barley spike is an indeterminate inflorescence and does not terminate in a single spikelet as in wheat. Within limits a certain amount of response to growth conditions is made at the tip of the barley spike in the number of fertile spikelets. Some response can be made at the base of the spike, but the capacity for responding at this point is much more limited.

Although the lemma differentiates before the awn, the awn grows more rapidly. It is not until well along in the development of the spikelet that the lemma and palea become long enough to enclose the anthers and other flower parts. A possible explanation for this behavior is suggested by Kennedy [4], who states that in the spikelet the awn corresponds to the leaf blade and that part of the glume below the insertion of the awn may be regarded as corresponding to the sheath of the leaf. He also states that of the three parts of the leaf the sheath develops last by intercalary growth which pushes up the blade. If the leaf parts and the spikelet parts are homologous as stated, then the slow growth of the lemma is in accord with development of the leaf sheath.

Up to the time that the anthers begin to differentiate, so far as the

[4] KENNEDY, P. B. THE STRUCTURES OF THE CARYOPSIS OF GRASSES WITH REFERENCE TO THEIR MORPHOLOGY AND CLASSIFICATION. U. S. Dept. Agr., Div. Agrostology Bul. 19, 44 pp., illus. 1899.

varieties used were concerned, no difference could be noted between a spike of a 2-row and a spike of a 6-row barley. As development continues the discrepancy in the development of the central and side spikelets of the 2-row barley becomes more apparent. The side spikelets develop very slowly, remain rudimentary without awns and infertile. On the other hand, while the side spikelets of the 6-row barley are always slower in development than the central spikelets, they finally attain nearly the same size, have awns, and are fertile.

SUMMARY

A study was made of the morphological development of the spike of a 2-row and a 6-row barley by dissecting the growing points from the stems. Photomicrographs of the various stages are shown.

Stem development from germination to pollination can be divided into two phases in each of which the growth response of the stem and growing point are different. In the first phase the internodes of the stem remain short, the growing point produces only leaf initials, and the undifferentiated portion of the growing point elongates. The beginning of the second phase is marked by the elongation of the internodes of the stem and the appearance of double ridges on the growing point. In the second phase the internodes of the stem elongate and the spike and its parts differentiate and develop.

The order of differentiation of the various parts of the spike as far as could be seen in this study are: Spikelet initials, empty glumes, lemma, palea, anthers, awn, and pistil.

Early differences in the time and rate of differentiation of the spikelets in the different parts of the spike are maintained and account for some of the variation in size among the spikelets of the mature spike.

The barley spike is an indeterminate inflorescence, and with the number of spikelets at each joint of the rachis limited, some response to the environment can be made in the number of fertile spikelets at the tip of the spike.

THE SEQUENCE OF APPEARANCE, MOLT, AND REPLACEMENT OF THE JUVENILE REMIGES OF SOME DOMESTIC BIRDS[1]

By D. C. WARREN, *poultry geneticist*, and C. D. GORDON, *graduate assistant*, *Kansas Agricultural Experiment Station*

PREVIOUS STUDIES

Interest in the problem discussed in this paper was first aroused in the authors by the occurrence of a heritable variation influencing the time of development of some of the remiges (flight feathers) in a flock of White Leghorns. A comparison of the sequence of appearance of the remiges in the chick of a normal and mutant stock (known as "retarded") led to findings regarding the order of appearance and the number of molts of these flight feathers which were not in agreement with previous records found in the literature. While this work was in progress Dunn and Landauer (*1*)[2] gave a detailed account of the sequence of replacement of the juvenile remiges in the Silver Spangled Hamburg fowl which the writers were able to confirm in studies on White Leghorns. A brief abstract of the data on the sequence of molt of the remiges in White Leghorns has been published (*8*).

The observations were later extended to other domestic birds to determine whether the regular manner of molt with respect to the members of the *Gallus* group but irregular with respect to the position of the feathers was found in other genera.

The work of Dwight (*2*) and Heinroth (*3*) indicate that some variability occurs in the sequence of molt of the secondary flight feathers of different species of passerine birds. Stone (*7*) also found a considerable lack of agreement among smaller land birds as to the order of molt of the flight feathers. The description by Rice, Nixon, and Rogers (*6*) of the sequence and number of molts of the remiges in White Leghorns does not agree with the writers' findings. It should be kept in mind that most of the observations cited on molt in wild birds were made on adults, whereas the present study is concerned primarily with the juvenile condition. The writers' studies indicate that the sequence of molt in gallinaceous birds differs somewhat in the juvenile condition from that reported by Marble (*4*) for the adult of *Gallus domesticus*. Marble (*5*) found it impossible to forecast the age at sexual maturity in White Leghorn females by the development of the primary wing feathers at 8 weeks of age.

METHODS AND MATERIAL

All of the species used in this study were a part of the stock carried at the Kansas Agricultural Experiment Station. As is indicated in the tabular data, the intervals between observations on the sequence of appearance and dropping of the remiges were varied in the different

[1] Received for publication Apr. 15, 1935; issued December 1935. Contribution no. 87, Department of Poultry Husbandry, Kansas Agricultural Experiment Station.

[2] Reference is made by number (italic) to Literature Cited, p. 470.

forms. As experience was gained the interval could be lengthened with less danger of confusion between new and old feathers. The use of dyes on the tips of the remiges aided in the recognition of the various molts. This is especially desirable in the early adult stage when members of three different sets of remiges may be present at one time.

In identifying the various remiges they were numbered anteriorly in the primaries and posteriorly in the secondaries. The writers have followed the lead of the poultrymen and called the first feather posterior to the primaries the axial rather than the first secondary. Its small size and distinctive behavior in time of appearance and molt would seem to justify placing it in a separate category. Since the secondaries appear irregularly there may be some difficulty for one inexperienced in identifying the different remiges. However, a close examination of the skin in which the remiges occur will usually solve the problem since the irregularly appearing axial and secondary no. 1 may be seen while yet embedded in the skin. The determination of the position of these two feathers will usually make it possible to identify accurately the other feathers at an early stage.

NUMBER OF REMIGES

In the various species studied the number of primaries was more constant than that of the secondaries. The forms studied—Single Comb White Leghorn, Single Comb Rhode Island Red, and Light Brahma chickens (*Gallus domesticus*); Pearl guineas (*Numida meleagris*); and Bronze turkeys (*Meleagris gallopavo*)—all showed 10 for the normal number of primaries. An extra primary was occasionally found in the various breeds of chickens observed. The eleventh primary occurred more frequently in the Light Brahmas and Rhode Island Reds than in the White Leghorns.

There was considerable variability in the number of secondaries both among breeds and individuals of a breed. The more usual number in White Leghorns was 14, in Rhode Island Reds 15, in Light Brahmas 16, in guineas 14, and in turkeys 16. The inner few secondaries were so reduced in size that they were not sharply differentiated from the tertiaries. In most cases data were taken only on the first 12 secondaries.

SEQUENCE OF EMERGENCE AND MOLT OF REMIGES

SINGLE COMB WHITE LEGHORN

The most extensive observations were made on Single Comb White Leghorns. Figure 1 shows the outline of the wing of an adult White Leghorn female indicating the numbers by which the remiges were identified in this study. Primaries nos. 1 to 7 and secondaries nos. 2 to 8 were usually present at hatching. Although they had grown several millimeters in length at time of hatching these feathers remained in their enclosing sheaths for several days. Progressing anteriorly in the primaries and posteriorly in the secondaries the new feathers appeared regularly as the chick aged. The axial and secondary no. 1 appeared late and there was considerable variability among individuals as to the age at which these feathers grew out. Figure 2 presents in graphic form the record of the mean age at which

the various remiges appeared and molted. Since the molted feathers were replaced immediately no distinction was made between time of molt and replacement.

The primaries which were not present at time of hatching appeared in very regular order at 10- to 14-day intervals. The intervals between appearance of the successive inner secondaries (nos. 8 to 12) were less uniform but the sequence was regular. The secondary no. 1 did not appear until the chick was about 2 weeks of age or not until the approximate time of the appearance of the tenth secondary and eighth primary. The axial feather did not emerge from the skin until about 4 weeks of age. This was the approximate time at which the twelfth secondary and the ninth primary appeared. It will be noted from figure 2 that the axial appeared only slightly before the first primary (no. 1) was molted.

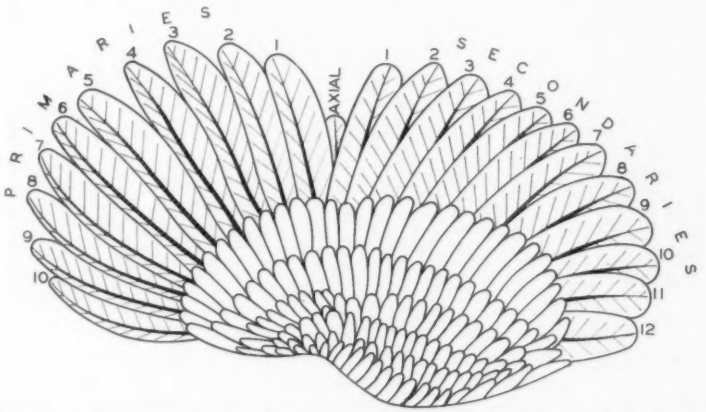


FIGURE 1.—Wing of a White Leghorn female showing system of numbering for the identification of the remiges. Only 12 secondaries are shown in this drawing.

THE FIRST MOLT

Beginning with feather no. 1, molt in the primaries progressed anteriorly in a very regular order. The secondaries were also molted in exactly the same order in which they appeared, beginning with no. 2 and being dropped in a regular manner from the outside in, or in the reverse order of the primaries. The secondary no. 1 and the axial were dropped at a much later date.

In practically all instances the corresponding flight feathers in the females appeared at a slightly earlier age than did those of the males. This was true of the molting of the remiges as well as of the time of their appearance. Since the number of secondaries was somewhat variable the observation on growth and molt included only feathers numbered 1 to 12. It is of interest to note from figure 2 that the time of emergence of the secondary no. 1 and the axial form a straight line with the series of the second set of primary feathers, the secondary no. 1 being at the top of the line and the primary no. 10 at the bottom. The same relationship was observed to hold between the

second secondary no. 1 and axial feather and the third set of primaries. It is not known whether this agreement of the behavior of these two feathers and the group of primaries has any significance.

Sequence of Juvenile and Adult Molt

Marble (*4*) has published a detailed account of the order of molt of the remiges in the adult fowl. In some respects there are close agreements of conditions found in the adult and the growing chick, while in other phases there is a rather wide divergence in sequence of molt. The first chick molt and the adult molt following a period of production agree in retention of the axial and secondary no. 1 until

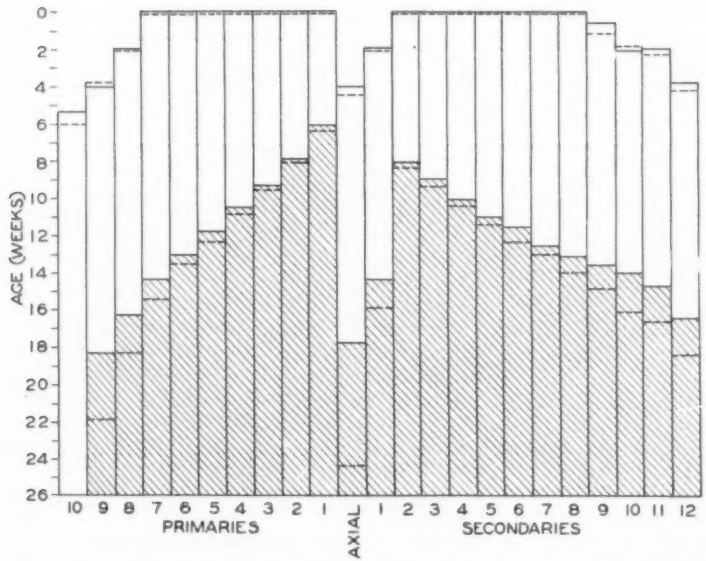


Figure 2.—Age at emergence and at molt of each remex of the White Leghorn. The upper outline of the graph indicates the age at which the remiges emerged. Those shown on the zero line were present at hatching. The lower gradations (diagonally lined) record the age at the time of molt of the remiges. The solid line is for females and the broken line for males. The space between the two solid or the two broken lines (of upper and lower graph) measures the period over which the feather was held before being molted.

the molt of the primaries and remaining secondaries is practically complete. There is a disagreement in that the inner small secondaries are the first to be molted in the adult while they are the last of the series of secondaries to be dropped by the chick. In both the chick and adult the usual procedure in the primaries is for them to be molted in regular order, beginning with the one (no. 1) adjoining the axial.

In a group of individuals of any one breed there was close agreement as to the age at which the various primaries and secondaries appeared and were dropped. This fact is well brought out in table 1. The data for the entire group of White Leghorns, presented in this table, show that the limits of time over which they varied in molting

any one flight feather seldom exceeded 2 weeks and that most of the feathers were dropped within the period of a week. The greatest variability occurred in the late-appearing axial, secondary no. 1, the smaller inner secondaries, and the tenth primary. The original tenth primary was frequently carried throughout much of the first adult year. Such birds can be recognized by the narrow, pointed, and much-curved appearance of this juvenile feather in the wing. Marble (*5*) reported that pullets carrying the original tenth primary over into the first laying year showed a tendency to mature at an early age.

TABLE 1.—*Age distribution for the emergence and molt of remiges in 15 White Leghorn females*

Age (weeks)	Birds having or molting primary no.—										Birds having or molting axial	Birds having or molting secondary no.—												
	10	9	8	7	6	5	4	3	2	1		1	2	3	4	5	6	7	8	9	10	11	12	13	14
0				15	15	15	15	15	15				15	15	15	15	15	15	15	12	1				
2			15									15								3	14	15	2		
4	5	15									15												12	4	
6	10								1	15													1	8	
8								5	14				14	8										2	1
10						2	12	10					1	7	14	8	4							1	6
12					7	13	3					1			1	7	11	12	7	4	2				7
14				12	8							11						3	8	10	11	10			1
16			12	3							4	3								1	2	5	11	6	4
18	1	14	3								8												4	6	8
20	2	1									2													3	3
22	7										1														
24	1																								

THE SECOND MOLT

In White Leghorns the usual procedure was for only one complete molt of the flight feathers to take place before sexual maturity was reached. In some cases the first molt was not complete at maturity because of the carry-over of the original outer primary. In practically all males examined and in some females there occurred a second molt. This molt was usually started at about 5 or 6 months of age. The sequence was identical with that of the earlier molt beginning at primary no. 1 and secondary no. 2, progressing outward in the primaries and inward in the secondaries. As in the preceding molt, the first primary to be dropped was released a little before the first secondary. In a group of 23 White Leghorn males studied, primary no. 1 was dropped in the second molt at the mean age of 23 weeks, secondary no. 2 at 27 weeks, primary no. 2 at 28 weeks, secondary no. 3 at 30 weeks, secondary no. 4 at 32 weeks, secondary no. 5 and primary no. 3 at 34 weeks, secondary no. 6 at 37 weeks, secondary nos. 7 and 8 at 39 weeks, and primary no. 4 at 40 weeks. Observations were discontinued at this age. Observations regarding the second molt were carried out on only a few females, but up to 36 weeks the molt had progressed as far as the fifth primary and the eighth secondary in some birds. The females were laying throughout this molt. In another group of 15 White Leghorns the second molt was started at 25 weeks of age, but the females showed no evidence of this molt at the termination of the observation when 34 weeks of age.

RHODE ISLAND REDS

As is seen from figure 3, the general sequence of emergence and molt of the remiges in Rhode Island Reds was very similar to that in White Leghorns. However, there were fewer flight feathers present at hatching than in White Leghorns and considerabley more variability as to the age at which the missing feathers appeared. Comparisons of the time of emergence and molt of the remiges in the various breeds and species studied are shown in table 2. The records on the 20 Rhode Island Reds were taken at 1 day and at 2, 4, 6, 8, 12, 16, 20, and 24 weeks. In the slower feathering individuals

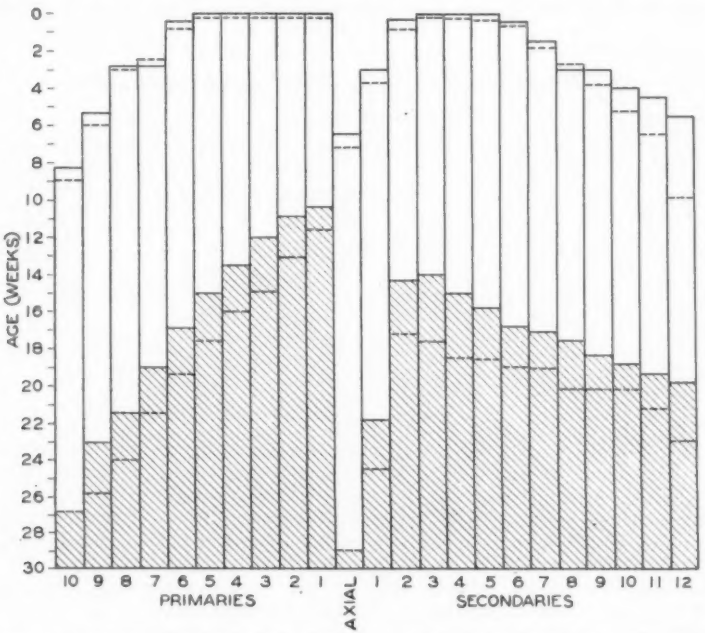


FIGURE 3.—Age at emergence and at molt of each remex of the Rhode Island Reds. The upper outline of the graph indicates the age at which the remiges emerged. Those shown on the zero line were present at hatching. The lower gradations (diagonally lined) record the age at the time of molt of the remiges. The solid line is for females and the broken line for males. The space between the two solid or the two broken lines (of upper and lower graph) measures the period over which the feather was held before being molted.

of the Rhode Island Reds and Light Brahmas the covert feathers appear before the remiges and may cause some confusion as to time of appearance of remiges. The first molt, although following the same order as in White Leghorns, was started and completed when the birds were about 6 weeks older. In no case had the axial molted at 6 months of age, and in several birds the original secondary no. 1 had not molted at this age, which was near sexual maturity. Both primary nos. 9 and 10 were the original feathers in most of the males at 6 months of age.

TABLE 2.—*Comparison of several breeds and species of domestic birds in respect to the mean age at which the first remiges appeared*

Feather no.	White Leghorn[1]		Rhode Island Red[2]	
	Female	Male	Female	Male
Primaries:				
1	1.0 day	1.0 day	1.0 day	1.0 day
2	1.0 day	1.0 day	1.0 day	1.0 day
3	1.0 day	1.0 day	1.0 day	1.0 day
4	1.0 day	1.0 day	1.0 day	1.0 day
5	1.0 day	1.0 day	1.0 day	.2 weeks
6	1.0 day	1.0 day	.5 weeks	.8 weeks
7	1.0 day	1.0 day	2.8 weeks	2.5 weeks
8	2.0 weeks	2.0 weeks	2.8 weeks	3.0 weeks
9	4.0 weeks	3.8 weeks	5.3 weeks	6.0 weeks
10	5.3 weeks	6.0 weeks	8.3 weeks	8.9 weeks
Secondaries:				
Axial	4.0 weeks	4.3 weeks	6.5 weeks	7.2 weeks
1	2.0 weeks	2.0 weeks	3.0 weeks	3.7 weeks
2	1.0 day	1.0 day	.3 weeks	.8 weeks
3	1.0 day	1.0 day	1.0 day	1.0 day
4	1.0 day	1.0 day	1.0 day	.2 weeks
5	1.0 day	1.0 day	1.0 day	.3 weeks
6	1.0 day	1.0 day	.5 weeks	.5 weeks
7	1.0 day	1.0 day	1.5 weeks	1.8 weeks
8	1.0 day	1.0 day	3.0 weeks	2.7 weeks
9	.4 weeks	1.0 weeks	3.0 weeks	3.8 weeks
10	1.9 weeks	1.8 weeks	4.0 weeks	5.2 weeks
11	2.0 weeks	2.0 weeks	4.5 weeks	6.5 weeks
12	3.9 weeks	4.0 weeks	5.5 weeks	9.8 weeks

Feather no.	Light Brahma[3]		Bronze turkey,[4] females and males[6]	Pearl guinea[5]	
	Female[6]	Male[6]		Female[6]	Male[6]
Primaries:					
1	(?)	(?)	(?)	(?)	(?)
2	(?)	(?)	(?)	(?)	(?)
3	(?)	(?)	(?)	(?)	(?)
4	(?)	(?)	(?)	(?)	(?)
5	(?)	(?)	(?)	(?)	(?)
6	(?)	(?)	(?)	(?)	(?)
7	2.6 weeks	3.8 weeks	(?)	(?)	(?)
8	4.0 weeks	5.1 weeks	(?)	3.6 weeks	3.7 weeks
9	5.2 weeks	6.8 weeks	5.9 weeks	4.0 weeks	4.2 weeks
10	8.0 weeks	10.9 weeks	7.7 weeks	6.0 weeks	6.0 weeks
Secondaries:					
Axial	6.2 weeks	6.0 weeks	5.0 weeks	4.0 weeks	4.0 weeks
1	4.6 weeks	4.7 weeks	4.0 weeks	(?)	(?)
2	2.4 weeks	2.9 weeks	(?)	(?)	(?)
3	(?)	2.9 weeks	(?)	(?)	(?)
4	(?)	2.7 weeks	(?)	(?)	(?)
5	(?)	(?)	(?)	(?)	(?)
6	(?)	(?)	(?)	(?)	(?)
7	(?)	(?)	(?)	(?)	(?)
8	2.4 weeks	3.1 weeks	(?)	(?)	(?)
9	2.8 weeks	3.8 weeks	(?)	(?)	(?)
10	4.0 weeks	4.0 weeks	(?)	1.5 weeks	1.5 weeks
11	4.0 weeks	4.0 weeks	(?)	2.0 weeks	3.1 weeks
12	4.4 weeks	5.6 weeks	(?)	4.0 weeks	4.2 weeks

[1] White Leghorns examined at 1 day of age and at 2-week intervals thereafter to 24 weeks.
[2] Rhode Island Reds examined at 1 day of age and at 2-week intervals to 8 weeks and at 4-week intervals thereafter to 24 weeks.
[3] Light Brahmas examined at 2-week intervals to 8 weeks and at 4-week intervals thereafter to 24 weeks.
[4] Turkeys examined at 4, 6, 8, and 12 weeks of age.
[5] Guineas examined at 1, 4, 6, 8, and 12 weeks of age.
[6] The interrogation marks indicate that the feather was present at time of first examination; thus in the guinea females primaries nos. 1 to 5 were present when the first examination was made at 1 week of age, but it is not known exactly when they emerged.

LIGHT BRAHMAS

Twenty-one Light Brahmas were examined for the development of the remiges at 2, 4, 6, 8, 12, 16, 20, and 24 weeks of age. The females developed their various primaries at about the same age as

the Rhode Island Reds, but the Brahma males were slightly later in the development of these feathers than were the Reds. The secondaries developed very similarly in the Rhode Island Reds and Light Brahmas up to about the tenth feather. From the tenth secondary on the appearance of the successive feathers was much earlier in the Brahmas than in the Rhode Island Reds. This same tendency was observed in the first molt. The Light Brahma males showed little molt of the secondaries until the twentieth week at which time secondaries nos. 2 to 10 were found to be molted. In the Light Brahmas the tendency for several secondaries to be molted at one time was much more noticeable than in the other breeds of fowl examined. As in the case of the Rhode Island Reds, all Light Brahmas retained the original no. 10 primary at 24 weeks of age. This was also true of the axial in all males and most of the females.

GUINEAS

Twenty guineas were examined for development of the remiges at 1, 4, 6, 8, and 12 weeks of age. For number of remiges present at hatching and age of appearance of the additional ones, the guineas were similar to the White Leghorns (table 2). The first 7 primaries and 10 secondaries were present at the first examination made at 1 week of age. The observations were not made over so long a period as in the breeds of chickens, but the data were sufficient to indicate that the sequence and rate of molt was comparable to that in White Leghorns. In figure 4 are given the data for comparison of rate and sequence of molt in the various species studied. The failure of the axial and secondary no. 1 to appear and molt in order with the contiguous feathers was observed here as was the case in the three breeds of chickens studied. The remiges of the female guineas appeared and molted slightly ahead of those of the males, as was true of the breeds of chickens observed.

TURKEYS

Observations were made on 18 turkeys at 4, 6, 8, and 12 weeks. The data secured provided little information as to the time of appearance of many of the remiges, but a comparison of those present at 4 weeks of age showed that they must have appeared at about the same age as did those of the White Leghorns and guineas. Here again the axial and secondary no. 1 appeared much later than the adjoining secondaries, indicating that this irregularity is characteristic of many gallinaceous birds. A comparison of the age at which the remiges emerged is found in table 2. In some forms records were not taken at hatching and the interrogation marks indicate that the feather was present at the first examination. In figure 4 is a comparison of the time of first molt for the various remiges. In making the comparison note should be made of the differences in age at the last examination in the different species. The graphs for the turkeys and guineas represent only the upper segment of the ones for the different breeds of chickens.

MOLT OF EXTRA REMIGES

It is of interest to consider the time of appearance and molt of the supernumerary remiges in the birds studied. The number of secondaries was somewhat variable and it was difficult to say what were extra

feathers in this group. It is true, however, that beginning with the secondary no. 2, feathers of this group were dropped in a regular order from the outside in, so any supernumerary feathers were dropped following the regular series.

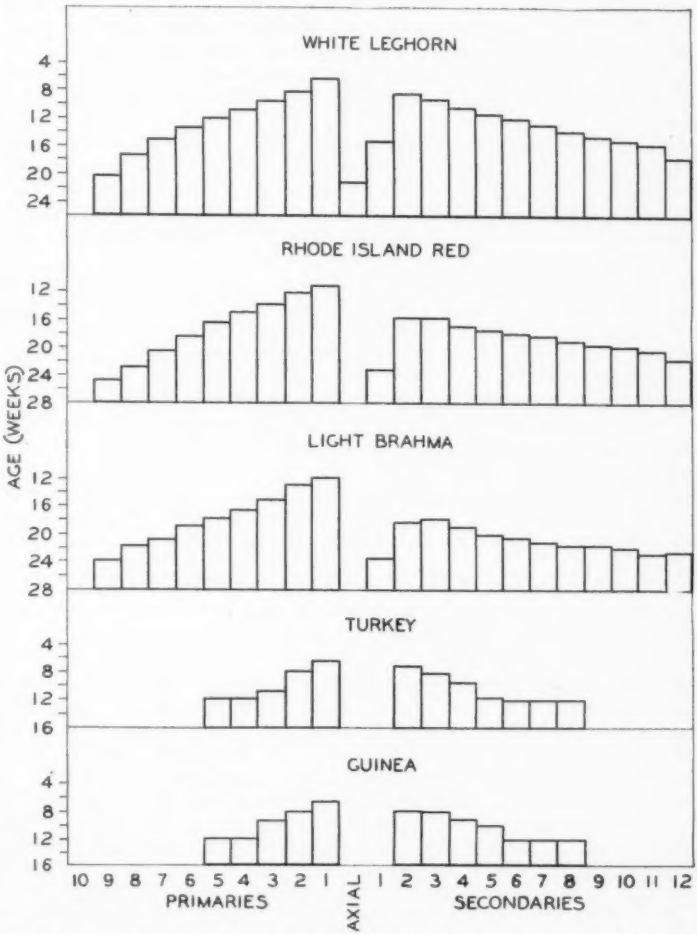


Figure 4.—Comparison of the age at, and sequence of, molt of the remiges in several species of domestic birds. Note that observations cover a much longer period in some species than in others, thus some graphs include only sections of the data given in others. Data are for mean of the two sexes.

In the case of the primaries the number seemed to be well established at 10. An occasional bird in all three breeds of chickens studied had 11. The eleventh feather always emerged after the tenth and was molted in the same order.

TIME OF MOLT AND RATE OF FEATHER GROWTH

There is little known as to the causal factors for molt in adults and the same is true of the juvenile molts. It is noted that although there are several primaries and secondaries present at time of hatching these feathers are not dropped at the same time. They are molted in a fixed and regular sequence. Those remiges appearing after hatching are molted in the same order in which they appear. This might indicate that the order of molt of the feathers present at hatching bears some relation to the sequence of their development in the embryo.

In order to determine whether a feather is molted after reaching a definite age, calculation was made of the period over which each of the original remiges was held. Since several feathers were present at hatching and since these feathers were molted in order with relation to position it would be expected that for those feathers present at hatching there would be a gradual increase in time held. Some idea of the relative periods over which the different remiges were held is gained by an examination of figure 2. The upper clear portion of each column indicates the length of period over which each original feather was held. The upper horizontal line marks the time of emergence and the lower one the time of molt. From table 3 it is seen that for primaries 1 to 7 and secondaries 2 to 8 the period increased by about 1 week for each successive feather in White Leghorns. Those appearing irregularly were found to vary considerably in period over which they were held, and the period was somewhat longer than for the other feathers Those feathers which were molted regularly and which appeared after hatching were held for about 12 weeks in the females and slightly longer in the males.

The growth rate of the various remiges is of interest in view of the variation in age at which they appear and in relative length in the adult. Table 4 gives the length of each remex (first set) at 3 weeks after it appeared. The age at which the various remex lengths were recorded varied, depending on the age at which the feather appeared. The mean lengths are for the respective feathers in 11 female and 8 male White Leghorns. Measurements were taken at weekly intervals, but the data in table 4 are the means for the third (weekly) record. The number of feathers measured was not large enough to supply very conclusive results. Nevertheless, the data indicate that the rate of growth of the feathers bears no relationship to the time at which they emerge. There is some tendency toward an agreement between the position and rate of growth of the secondaries. Beginning with the axial feather, there is a somewhat regular decrease in rate of growth if we exclude the twelfth feather. An examination of figure 1 shows no correspondence between the adult length of the feather and its rate of growth. In considering the results in table 4, it should be kept in mind that secondary no. 1 does not appear until about the time that secondary no. 10 appears, and that the axial feather emerges along with the twelfth secondary. Secondaries 7 to 11 averaged somewhat shorter than the others after 3 weeks' growth. The axial and secondary no. 1 in both males and females showed considerably more rapid growth than did other remiges and these feathers were also considerably delayed in appearing.

TABLE 3.—*Period (weeks) between emergence and molt of the first set of remiges in White Leghorns*

Feather no.	Female		Male		Feather no.	Female		Male	
	Primaries	Secondaries	Primaries	Secondaries		Primaries	Secondaries	Primaries	Secondaries
Axial	-------	13.7	-------	20.0	7	14.4	12.4	15.5	13.0
1	6.0	12.3	6.3	13.8	8	14.3	13.1	16.3	14.0
2	7.9	8.1	8.0	8.3	9	14.3	13.2	18.0	13.8
3	9.3	8.9	9.5	9.3	10	16.1	12.1	19.3	14.2
4	10.4	10.1	10.8	10.3	11	-------	12.7	-------	14.5
5	11.7	10.9	12.3	11.3	12	-------	12.5	-------	14.3
6	13.1	11.5	13.5	12.3					

TABLE 4.—*Comparison of lengths (millimeters) of remiges at the end of the first 3 weeks of growth in White Leghorns*

Sex and chick no.	Length of primary no.—										Length of axial	Length of secondary no.—										
	10	9	8	7	6	5	4	3	2	1		1	2	3	4	5	6	7	8	9	10	11	12
Female:																							
923	69	73	...	78	85	87	87	83	78	66	92	78	72	74	74	74	73	70	62	63	67	71	73
924	66	75	63	64	68	72	74	73	70	60	87	76	65	64	63	60	61	60	57	58	55	60	72
927	69	81	63	69	75	75	74	78	75	68	99	60	68	68	67	65	66	61	56	58	49	58	...
928	78	89	66	75	82	85	83	82	...	78	86	95	74	73	72	73	71	67	54	58	58	68	74
929	78	88	...	75	81	83	83	83	78	76	90	93	73	73	73	70	70	68	63	58	58	54	52
930	66	80	68	71	75	76	77	77	76	68	88	89	68	68	68	67	67	63	60	53	63	51	84
935	75	84	71	76	84	85	85	...	82	75	78	94	76	75	...	73	71	70	65	60	64	54	70
937	70	80	66	68	76	73	77	77	73	68	80	82	66	66	65	64	64	60	56	50	55	60	60
943	80	70	...	74	77	80	80	80	80	70	86	78	70	70	68	69	68	64	63	60	64	66	66
944	81	68	...	74	80	82	81	80	77	67	75	81	73	72	71	71	69	65	64	58	61	71	70
945	...	74	...	62	70	70	70	71	68	55	72	77	63	62	60	57	58	55	50	45	47	48	66
Mean	73	78	66	71	78	79	80	78	76	68	85	82	70	70	68	68	67	64	59	56	58	60	69
Male:																							
922	71	83	68	...	78	79	77	78	74	65	91	96	67	67	69	68	67	65	62	55	64	50	72
931	75	65	71	75	83	83	83	82	77	74	95	77	75	77	78	74	72	70	70	62	62	67	66
932	87	74	...	77	84	84	84	82	80	71	102	76	73	75	73	73	73	71	68	62	68	72	74
933	77	61	88	75	82	86	85	85	82	85	95	96	72	72	70	...	62	60	63	73	62	62	...
934	70	59	86	71	76	81	82	82	80	72	85	87	70	70	68	67	58	62	57	70	57	61	66
938	...	88	62	52	63	63	67	69	66	60	96	92	79	57	53	...	48	45	38	30	...	...	65
941	...	72	78	63	68	71	73	69	68	66	-------	72	62	62	63	62	58	56	48	38	48	72	72
946	...	...	65	76	82	83	83	83	82	74	-------	...	74	74	74	72	71	67	65	55	59	...	...
Mean	76	72	74	70	77	79	79	79	76	70	94	85	72	69	69	69	64	62	59	56	60	62	69

SUMMARY

Three breeds of chickens (White Leghorn, Rhode Island Red, and Light Brahma), guineas, and turkeys all agree in having several primaries and secondaries present at hatching. In the White Leghorns, turkeys, and guineas more feathers were present at hatching and the missing feathers appeared earlier than in the Rhode Island Reds and Light Brahmas.

The irregular sequence of emergence and molt of the secondaries was found to be characteristic of all species of domestic birds examined.

One complete molt of the remiges usually took place during growth, but frequently the original primary no. 10 was carried unmolted by the sexually mature chicken.

A second molt, following the same sequence as the original one, was observed in some chickens when they approached sexual maturity.

There was considerable difference in period of time over which the various remiges were held before being molted.

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MACROSPOROGENESIS AND EMBRYOLOGY OF MEDICAGO[1]

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INTRODUCTION

This study of the macrosporogenesis and embryology of alfalfa (*Medicago sativa* L.) is preliminary to an investigation of the causes of the failure of seed setting in certain strains of this species. Reeves (*13*)[2] gave a detailed account of microsporogenesis and traced the development of the ovule and of the embryo sac (*14*). He did not give the details of macrosporogenesis and did not continue the study through fertilization and the development of the embryo. Martin (*11*) described stages in the development of the embryo sac of alfalfa and compared these with corresponding stages in other Leguminosae. Ghimpu (*7*), Kawakami (*10*), Tschechow (*17*), Reeves (*13*), and Fryer (*6*) have reported the chromosome number of *M. sativa* as $n=16$, $2n=32$.

MATERIAL AND METHODS

The material for this investigation was collected from plants growing in the greenhouses of the Department of Genetics, Wisconsin Agricultural Expermient Station, during the late winters and early springs of 1932–34. These plants were producing an abundant crop of seed. For comparative purposes, material of seven other species of *Medicago*—*M. hemicycla*, *M. glutinosa*, *M. falcata*, *M. platycarpa*, *M. ruthenica*, *M. lupulina*, and *M. dzawakhetica*—was also collected. The first three have the same chromosome number as *M. sativa* and the remainder have half that number. Buds, young flowers, and fruits of various ages were placed in the following fixatives: La Cour's, Licent's, and Karpechenko's modification of Navashin's fluid. The best preparations were obtained from the material fixed in the two fixatives last named.

OBSERVATIONS

MACROSPOROGENESIS

Each ovule of *Medicago sativa* contains usually 2 or 3 primary sporogenous cells. These lie, in most instances, side by side in the nucellus (pl. 1, *A*), but occasionally they are end to end, appearing as a tetrad much as was described by Reeves (*14*). The primary sporogenous cells are easily identified by their large size and peculiar staining properties. They become more or less deeply embedded in the nucellus in consequence of some further division of the cells of the hypodermal layer. The presence of more than one sporogenous

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[2] Reference is made by number (italic) to Literature Cited, p. 476.

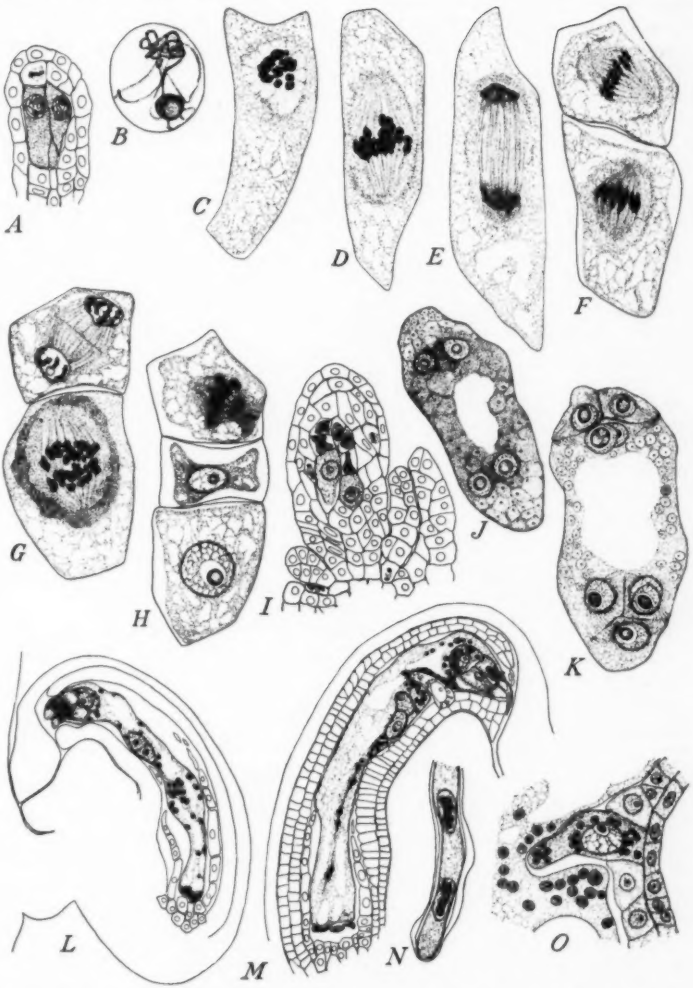
cell in *M. sativa* was noted by Martin (*11*) and by Reeves (*14*). The members of this genus vary in this respect. Guignard (*9*) found a single sporogenous cell in *M. arborea*. Corti (*4*) found a like condition in *M. hispida* and *M. arabica*, although occasionally more than one primary sporogenous cell was seen. There are usually two or more sporogenous cells in *M. dzawakhetica*, *M. falcata*, *M. glutinosa*, and *M. hemicycla*. One such cell is the rule in *M. lupulina*, *M. platycarpa*, and *M. ruthenica*, although in an occasional ovule two or more sporogenous cells are present.

The primary sporogenous cell functions as the macrospore mother cell without further division. It increases in size until it is about three times as long as broad. While the nucleus of the spore mother cell is passing through the early stages of the heterotypic division (pl. 1, *B*, *C*), the cytoplasm remains finely vacuolate. As meiosis progresses the cytoplasm at the ends of the cell becomes more vacuolate, and a dense zone surrounds the heterotypic and later the homeotypic spindles (pl. 1, *D–G*). The 16 pairs of chromosomes as seen at the multipolar spindle stage (pl. 1, *C*) and on both the heterotypic and homeotypic equatorial plates vary in size and shape. One pair is particularly prominent because of its large size. After the completion of the heterotypic division, a cell plate is formed and the macrospore mother cell is divided in such a manner that the chalazal daughter cell is about twice as large as the micropylar cell.

The axis of the homeotypic spindle in the chalazal cell is usually longitudinal, whereas the spindle in the micropylar cell may be transverse (pl. 1, *F*, *G*). In some instances they are formed simultaneously, in others the nuclear division in the chalazal cell either precedes or follows that in the sister cell (pl. 1, *G*, *H*). Occasional figures show an abortive equatorial plate in a micropylar cell which has already begun to disintegrate. Probably the occasionally observed row of 3 instead of 4 macrospores arises in this way. Most observers have found a linear row of 4 macrospores in the Leguminosae. In a few species, however, a row of 3 macrospores has been described. Guignard (*8*, *9*) noted the formation of only 3 in *Phaseolus multiflorus* and in *Medicago arborea*. Weinstein (*19*) found the presence of 3 macrospores to be typical of *P. vulgaris*. This was brought about as the result of an abortive homeotypic division in the micropylar cell.

The spore mother cell, or in some instances more than one of the spore mother cells, of the apical ovule passes through these divisions first; corresponding divisions follow successively toward the base of the ovary. Microsporogenesis likewise is initiated in those stamens at the apex of the bud and gradually advances toward its base. Thus microspore tetrads may be found in stamens in the apical region of the bud, heterotypic spindles being present at the same time in the more basal stamens. In a few instances heterotypic spindles were found in apical ovules of buds in which the anthers at the apex contained microspore tetrads. Close examination revealed the presence of a tetrad of macrospores in the ovule as well as a spore mother cell bearing a heterotypic spindle, indicating that macrosporogenesis may lag in some of the sporogenous cells.

In numerous cases 2 or more tetrads are formed in a single ovule (pl. 1, *I*), and a few ovules were observed to contain 2 well-developed gametophytes. The chalazal macrospore of the row of 3 or 4 becomes



A, Portion of nucellus showing three primary sporogenous cells, × 330; *B*, nucleus of macrospore mother cell, early spireme stage, × 1,365; *C*, macrospore mother cell, heterotypic division, multipolar spindle stage, × 1,365; *D*, same as *C* at early equatorial-plate stage, × 1,365; *E*, same as *C* at telophase, × 1,365; *F*, homeotypic equatorial-plate stage, × 1,365; *G*, homeotypic division, anaphase stage in chalazal cell and cell plate formation in micropylar cell, × 1,365; *H*, delayed mitosis in micropylar cell, large chalazal cell is functional macrospore, × 1,365; *I*, ovule with two tetrads, one being in a stage similar to that of *H*, × 330; *J*, 4-nucleate embryo sac showing remnants of spindles between nuclei and large central vacuole, × 1,365; *K*, 8-nucleate embryo sac showing cell-plate formation, × 1,365; *L*, ovule with maturing embryo sac, × 146; *M*, embryo sac showing entrance of a pollen tube, an integumentary cell extending into the sac, × 146; *N*, tip of pollen tube showing two male gamete cells, tube nucleus having disintegrated, × 1,365; *O*, detailed drawing in integumentary cell extending into embryo sac *M* (note active nucleus and starch grains in this cell), × 683. (Drawings made with Abbé camera lucida at table level.)

A, Egg apparatus and pollen tube shown in plate 1, *M*, × 725; *B*, egg, showing male gamete nucleus closely appressed to egg nucleus, × 683; *C*, stage in process of fusion of male gamete nucleus with egg nucleus, × 1,365; *D*, zygote nucleus at spireme stage, × 1,365; *E*, same as *D*, with two groups of chromosomes and two nucleoles present, × 1,365; *F*, same as *D*, later stage (note position of satellite chromosomes), × 1,365; *G*, chromosomes advancing to equatorial plate (note two groups), × 1,365; *H*, oblique view of early zygotic spindle, chromosomes at left having advanced further on to the equatorial plate than those at the right, × 1,365; *I*, lateral view of equatorial plate in zygote showing two groups of chromosomes, × 683; *J*, zygotic equatorial plate, polar view, × 1,365; *K*, same as *J*, lateral view, × 683; *L*, nuclear division in zygote, telophase, × 683; *M*, binucleate proembryo showing cell-plate formation with extra pollen tubes present, × 683; *N*, 2-celled proembryo, × 683; *O*, same as *N*, nucleus of apical cell dividing, × 683; *P*, stage in formation of 4-celled proembryo, × 330; *Q*, 4-celled proembryo, × 330; *R*, 5-celled proembryo, × 330; *S*, 6-celled proembryo, × 330; *T*, 2-celled embryo with 5-celled suspensor, × 330. (Drawings made with Abbé camera lucida at table level.)

the functional embryo-sac mother cell and the other spores disintegrate. This is in agreement with the reports of Martin (*11*) and Reeves (*14*). In *Medicago arborea* the lily type of embryo-sac development, wherein all 4 macrospore nuclei participate in the formation of the embryo sac, has been described by Guignard (*9*) and Herail, as quoted by Schnarf (*15*). An examination of the other species used for comparative purposes in the present study has shown that in each the chalazal spore alone develops into an embryo sac.

As a result of 3 nuclear divisions an 8-nucleate embryo sac is formed. The spindles of the second division apparently persist (pl. 1, *J*) so that after the last division the 4 nuclei at each end of the embryo sac are connected by spindle fibers (pl. 1, *K*). Cell plates are formed between the nuclei in such a manner that 3 of the nuclei at each end of the embryo sac are incorporated into a like number of cells, and the fourth nucleus remains in the central region. Thus a 7-celled embryo sac is formed consisting of an egg and 2 synergids at the micropylar end, 3 antipodal cells at the chalazal end, and, an elongated endosperm mother cell in the mid-region (pl. 1, *L*). The nucellus breaks down so that the micropylar end of the embryo sac comes to ie in direct contact with the inner integument. Mottier (*12*) observed cell-plate formation in the development of the embryo sac of *Lilium martagon*, and the writer (*3*) has described a similar phenomenon in *L. henryi*. Olive Rees (unpublished results) likewise observed cell-plate formation in the development of the embryo sac of *Solanum tuberosum*.

Small starch grains are to be observed in the four-nucleate embryo sac (pl. 1, *J*). They continue to increase in size and each grain has a definite shape at the time of the third nuclear division. Well-developed starch grains are abundant in the mature gametophyte.

The egg apparatus consists of three pear-shaped cells, the egg being somewhat larger than either synergid. A large vacuole appears in the basal region of each synergid, the small nucleus being embedded in the dense cytoplasm in the middle region of the cell. Just prior to fertilization the micropylar ends of the synergids elongate and extend into the micropyle. The cytoplasm of this region contains minute canals (filiform apparatus), which extend from the large basal vacuole toward the apex (pl. 2, *A*). The large egg nucleus is embedded in the dense cytoplasm in the basal region of the egg. This cell contains numerous starch grains, which are lacking in the synergids.

The antipodal cells, although usually well formed at the time of fertilization, disingegrate shortly thereafter. Reeves (*14*) has described early disintegration of the antipodal cells and finds that conductive cells, similar to the "Leitzellen" described by Ernst (*5*) for *Tulipa gesneriana*, extend before fertilization into the chalazal end of the embryo sac. No evidence of such structures has been found in the material used in this investigation.

The polar nuclei come to lie near each other in a region closely adjacent to the basal portion of the egg. They do not completely unite until the time of fertilization. Starch grains are abundant in the endosperm mother cell, especially in the dense cytoplasm near the fusion nuclei and near the egg apparatus. Comparatively few starch grains appear at the chalazal end of the endosperm mother cell and none in the antipodal cells (pl. 1, *M*).

FERTILIZATION AND DEVELOPMENT OF THE EMBRYO

Fertilization takes place between 24 and 27 hours after pollination. The pollen tube enters the embryo sac between the synergids and the egg. Usually only one pollen tube was seen, but in a few instances extra pollen tubes were present (pl. 2, *M*). The synergids are not broken down by the pollen tube as in *Phaseolus vulgaris* (Weinstein (*18*)), but persist for some time after fertilization.

The two male gemetes are discharged from the pollen tube in the vicinity of the egg. As is shown in plate 1, *N*, each gamete nucleus is surrounded by a layer of cytoplasm which is distinct from that of the pollen tube. The tube nucleus may disintegrate early, but in a few instances it was found near the apex of the pollen tube (pl. 2, *A*). In the process of fertilization one male gamete nucleus becomes closely appressed to the egg nucleus, and the other unites with the fusing polar nuclei. The male gamete nuclei are not rounded as in *Phaseolus* (Brown (*1*)), but are more elongate as in *Melilotus* (Cooper (*2*)). Each contains a small nucleolus near one end.

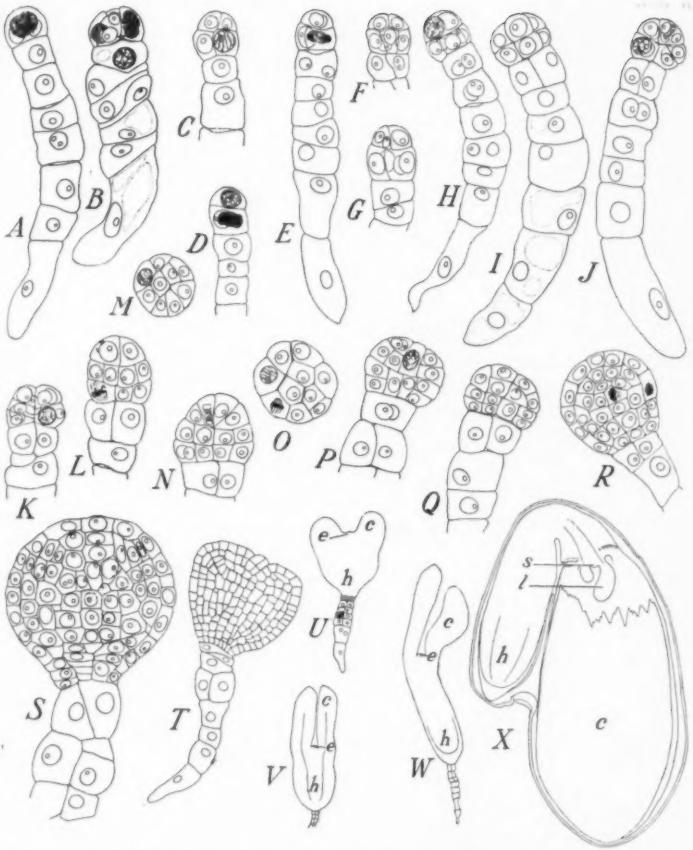
The small densely staining male gamete nucleus which is closely appressed to the less densely staining egg nucleus (pl. 2, *B*) now increases in size and its chromatic network tends to spread out so that ultimately the networks of the fusing nuclei are substantially similar (pl. 2, *C*) and the nuclear membranes disappear in the region of contact of the two nuclei. The nucleolus of the male gamete nucleus also enlarges to some extent but does not become as large as that of the egg nucleus.

Two distinct groups of chromosomes are now formed, one arising from the nuclear material of the male gamete and the other from that of the egg. These can be followed in plate 2, *D* to *G*. The two satellite chromosomes, one from each parent, are widely separated within the nucleus (pl. 2, *F*).

During the stages just described the fusion nucleus of the endosperm mother cell has divided once or sometimes twice, and by the time of the equatorial-plate stage in the division of the zygote nucleus commonly 2, and often 4, endosperm nuclei are present. The 2 chromosome groups of the zygote are usually somewhat separated on the equatorial plate (pl. 2, *G* to *I*), but occasionally the 2 groups cannot be distinguished (pl. 2, *J*, *K*).

The zygote divides transversely to form a two-celled proembryo consisting of an enlarged basal cell and a much smaller apical cell (pl. 2, *L* to *N*). Extra pollen tubes have been found in embryo sacs at this time. Plate 2, *M*, shows the two male gametes being discharged from a ruptured pollen tube.

The apical cell divides further so that a proembryo is formed consisting of 6 cells (pl. 2, *O*–*S*). Although both nuclei are preparing to divide in the 2-celled proembryo in plate 2, *N*, indicating that such a procedure perhaps may sometimes occur, usually only the apical cell divides (pl. 2, *P*). In one case, however, a mitotic figure was observed in the basal cell (pl. 2, *R*). According to Souèges (*16*), the apical cell of the 3-celled proembryo divides to form the true embryo in *Medicago lupulina*. Martin (*11*) found that it is the terminal cell of the 5-celled filament that forms the embryo in *M. sativa*. In all the material thus far examined a 6-celled proembryo is formed (pl. 2, *S*), and from the apical cell of such a proembryo the embryo is



A, Division forming 4-celled embryo, magnification here and unless otherwise noted, × 265; *B*, cells of 4-celled embryo dividing transversely; *C–H*, stages of embryo development wherein either the first or second division was transverse, showing suspensor increasing in size; *I*, 8-celled embryo with attached 8-celled suspensor; *J*, *K*, cell divisions leading to 16-celled stage; *L*, two layers formed from basal tier of cells with apical cells undivided; *M*, transverse section of basal tier of embryo showing periclinal divisions; *N*, embryo showing nuclear division in apical tier; *O*, somewhat later stage than in *N*; *P*, *Q*, transverse division of cells of apical tier; *R*, *S*, older embryos becoming more spherical in shape; *T*, still older embryo, flattened apically and showing first evidences of primordia of cotyledons and epicotyl, × 257; *U*, embryo with primordia of cotyledons (*c*), epicotyl (*e*), and hypocotyl (*h*), and suspensor at stage of greatest development, × 115; *V*, embryo with elongating cotyledons and hypocotyl, × 60; *W*, embryo bending in region of epicotyl, with suspensor disintegrating, × 60; *X*, mature embryo with portion of upper cotyledon cut away to show primordia of leaf (*l*) and stem tip (*s*), × 30. (Drawings made with Abbé camera lucida at table level.)

developed. The cells of the suspensor divide, and the suspensor increases in size.

Material from high seed-setting strains of alfalfa, collected at regular intervals after pollination shows considerable variation in embryo development. Stages in the division of the zygote and occasionally 2-celled proembryos are to be found 31 hours after pollination. The endosperm at this time contains from 2 to 4 nuclei. Two- to four-celled proembryos are present 48 hours after pollination, and 24 hours later these have so developed that they are composed of 5 or 6 cells. True embryos ranging from 2 to 16 cells in size are present 120 hours after pollination. Such variation in size of embryos is found not only between different ovaries, but also within a single ovary, where embryos were observed ranging from 4 to 12 cells in size.

Although there is an abundance of pollen tubes in the ovary at the time of fertilization, only about half or less of the ovules show the presence of proembryos at 31 and 48 hours after pollination. The cytoplasm of an embryo sac in which fertilization has not occurred remains apparently normal for a considerable time, the first evidences of disintegration being found in ovules collected 72 hours after pollination. This disintegration continues and ultimately the whole ovule becomes involved, so that at 120 hours after pollination the unfertilized ovules are small and very much shrunken. An examination of about a hundred ovaries revealed a range in number of ovules per ovary from 8 to 14 and a range in number of fertilized ovules per ovary from 1 to 6, the average being between 3 and 4.

The division of the apical cell in the formation of the embryo is usually parallel to the longitudinal axis of the proembryo (pl. 2, *T*). By two further divisions, the first vertical and the next transverse, an 8-celled embryo is formed (pl. 3, *A*, *B*, and *I*). Occasionally the first division is transverse and the second and third divisions are vertical (pl. 3, *C–H*). Periclinal divisions now occur which cut off the dermatogen (pl. 3, *I–L*). The cells of the embryo continue to multiply, forming first a spherical mass which later elongates and broadens at the apex (pl. 3, *M–T*). At this stage certain cell groups at opposite sides of the periphery of the apex as well as those in the basal region of the embryo become actively meristematic, and thus the cotyledons and hypocotyl are initiated (pl. 3, *T*, *U*).

The cotyledons appear as two outgrowths at the periphery of the apical region of the embryo. The epicotyl is a smaller outgrowth between them at the apex. The cotyledons elongate rapidly, becoming long, broad, and flattened (pl. 3, *V*). The hypocotyl also elongates, and during its further course of development the embryo curves in the region of the epicotyl (pl. 3, *W*) so that at maturity the cotyledons and hypocotyl are almost parallel (pl. 3, *X*). Provascular strands are differentiated early in the embryo (pl. 3, *V*). In the mature embryo, primordia of the first leaves are present near the apex of the stem. In the embryo represented in plate 3, *X*, a portion of one cotyledon is cut away to show the stem tip.

SUMMARY

Each ovule of *Medicago sativa* contains usually 2 or 3 primary sporogenous cells.

The primary sporogenous cells function directly as macrospore mother cells.

In consequence of the two meiotic divisions, the macrospore mother cell produces a row of four macrospores.

Occasionally only three cells are produced as a result of meiosis, the micropylar cell possessing an abortive homeotypic division spindle.

Usually 1, occasionally 2, and sometimes 3 macrospore tetrads are found in a single ovule.

The chalazal cell develops into an 8-nucleate, 7-celled embryo sac; the other macrospores disintegrate.

One embryo sac is usually formed in an ovule; occasionally two are present.

The apices of the synergids elongate into the micropylar canal, and a distinct filiform apparatus is present.

Fertilization takes place, under greenhouse conditions, between 24 and 27 hours after pollination.

The pollen tube enters the embryo sac between the synergids, neither of which disintegrates until later.

The antipodals persist for some time after fertilization.

In the course of gametic union, the chromosomes of the two gametes remain in two more or less distinct groups until the equatorial-plate stage of the first division of the zygote nucleus.

The zygote by transverse divisions forms a filament of six cells. The embryo develops from the terminal one of these cells. The other five cells by a few further divisions form the suspensor.

The embryo develops in a typical manner.

Although there is an abundance of pollen tubes present, less than half the ovules in an ovary show signs of fertilization. In heavy seed-setting lines of alfalfa there is an average of 3 to 4 seeds per pod, whereas 10 to 12 ovules are present in each ovary.

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